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SULFUR METABOLISM IN THE LUGWORM, ARENICOLA CRISTATA STIMPSON[1, 2]

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Among the published reports on invertebrate biochemistry is a study by Robin (1954) using *Arenicola marina* L. Robin hypothesized that taurocyamine, a sulfur-containing guanidine derivative, was formed from taurine. Although the hypothesis has not been demonstrated experimentally, it constitutes a major part of the information presently available on sulfur metabolism in invertebrates.

Taurine in invertebrates generally has been regarded as a metabolically inactive compound. In *Arenicola*, however, since taurocyamine appears to be the phosphagen involved in energy transfers related to muscular activity, Robin's hypothesis requires an active metabolic role for taurine. Relatively high levels of taurine have been reported from a diversity of marine invertebrates. The fact that no definite role has been demonstrated for these high levels suggests that an organism such as *Arenicola*, which presumably both synthesizes and metabolizes taurine, might be a useful experimental tool for studying sulfur metabolism in invertebrates.

The present study was undertaken to determine the biochemical pathway leading to taurine formation in *Arenicola cristata* Stimpson, and to determine whether taurocyamine is synthesized by the route suggested by Robin or by some other mechanism.

Materials and Methods

Animals

The specimens of *A. cristata* employed in this study were obtained from two sources. The Marine Biological Laboratory, Woods Hole, Massachusetts, supplied large specimens (50 to 200 gm. fresh weight). Worms of this size apparently are typical of the cold water environment in the Cape Cod area. Smaller individuals (5 to 20 gm. fresh weight) were obtained from the vicinity of St. Petersburg, Florida, through the courtesy of Dr. Victor G. Springer of the Florida State Board of Conservation Marine Laboratory.

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[2] The data presented here are extracted from the Ph.D. thesis of Walter Abbott.

[3] National Science Foundation Predoctoral Fellow in Biology, 1957–60.

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Compounds used

Taurocyamine was synthesized by reacting taurine with S-methylisothiourea in ammoniacal medium, according to the general method of Schütte (1943) as modified by Thoai and Robin (1954). Traces of taurine in the product were removed by chromatography on a column of Dowex 50 (H^+).

Hypotaurine (2-aminoethanesulfinic acid) was prepared by the method of Cavallini *et al.* (1955).

S^{35}-taurine was prepared from S^{35}-sodium sulfite (Abbott Laboratories) and β-bromoethylamine hydrobromide (Cortese, 1943). The product was purified by chromatography on a column of Dowex 50 (H^+).

S^{35}-methionine, S^{35}-cystine, and 3-C^{14}-serine were obtained from various commercial sources. S^{35}-cysteine was prepared by reduction of S^{35}-cystine (Lucas and Beveridge, 1940).

Extract preparation

Enzymatically active preparations were made by homogenizing living *A. cristata* with 10 volumes of 0.01 *M*, pH 7.3 phosphate buffer at temperatures of 0° to 4° C. Choice of pH was based on values given by Spector (1956) for *Arenicola* sp. The homogenates were centrifuged in the cold for 15 minutes at $1500 \times g$ to remove large amounts of fibrous debris, then, after decantation, for 30 minutes at $25{,}000 \times g$ to remove very small particulate components. The clear supernatant from the second centrifugation was quick-frozen immediately in Erlenmeyer flasks, and was lyophilized. A light, flesh-colored powder resulted. This powder was totally soluble in the phosphate buffer.

For determination of amino acids and related compounds, water-soluble fractions were prepared by homogenizing living worms in 80% ethanol and extracting by the method of Awapara (1948).

Before worms were used to make either type of extract, they were placed in enamel trays containing small amounts of clean sea water, in order to allow time for the worms to empty their digestive tracts. Attainment of this condition was assumed when fecal casts were not extruded for a period of at least 30 minutes.

Separation and identification of compounds

Dowex 50 (H^+), 200- to 400-mesh, a sulfonic acid-type cation exchanger, was used to separate polar compounds into two groups. Those compounds not retained on a column of the resin during elution with distilled water were termed "acidic compounds" in this study. Those compounds retained on the resin under these conditions were designated "basic compounds." Acidic compounds of interest here were taurine, taurocyamine, cysteinesulfinic acid, and cysteic acid. Basic compounds were displaced with 4 N NH_4OH.

For group separation, 3 gm. of Dowex 50 (H^+) were sufficient to treat an amount of water extract equivalent to at least 1 gm. fresh weight of tissue. In a small column were placed 1.5 gm. of resin. Another 1.5 gm. were slurried with the sample in a small beaker for 5 to 10 minutes. Contents of the beaker then were poured into the column. Beaker and column were rinsed liberally with distilled water.

Quantitative recovery of the acidic compounds was demonstrated by treating

individual standards of these compounds in the manner just described. Recoveries in excess of 95% were obtained in all cases when 15 standard samples were treated as just outlined.

Thompson and Morris (1959) reported the chromatographic separation of mixtures of amino acids on a column of Dowex 50 (Na^+), using water or aqueous ethanol as solvent. This suggested separation of acidic compounds from *Arenicola* by chromatography on a column of Dowex 50 (H^+), using distilled water for elution. The column, 60 × 0.9 cm., was prepared from a slurry of reagent grade Dowex 50 (H^+) which had previously been washed to remove excess acidity. A column was used only once. The sample to be resolved was placed in the column in a volume of 1 ml. Elution was carried out at a rate of 5 to 6 ml. per hour. One-milliliter fractions were collected. Mixtures of standards could not be resolved until a soluble inorganic salt was added to the sample (25 to 50 mg./ml. NaCl), whereupon resolution became excellent (Fig. 1). The

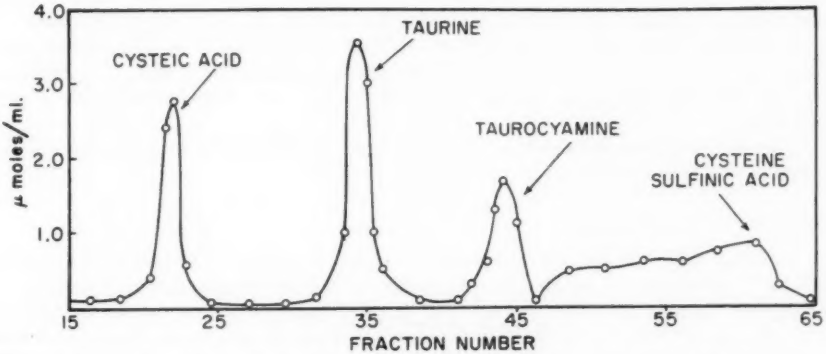

FIGURE 1. Elution of standards from 60 × 0.9 cm. Dowex 50 (H^+) column.

worm extracts contained sufficient inorganic material to obviate addition of salt. Schram and Crokaert (1957) obtained similar resolution of taurine and taurocyamine by eluting Dowex 50 (H^+) columns with 0.1 *M*, pH 2.5 citrate buffer.

The four acidic components could be resolved by one-dimensional paper chromatography with phenol: water (72% v/v) as solvent. The basic components were separated by two-dimensional paper chromatography with phenol: water and 2,4-lutidine: water (65% v/v) as solvents. Papers used were Whatman Nos. 1, 4, or 3 MM.

Quantitative determinations of amino compounds were carried out by the method of Landua and Awapara (1949). Guanidylated entities were determined colorimetrically with diacetyl and α-naphthol (Rosenberg, Ennor and Morrison, 1956). Location of spots on papers was accomplished by two methods. Amino compounds were found by treatment of papers with 0.025% ninhydrin in acetone. Guanidino compounds were detected with the diacetyl reagent of Smith (1958), using the reagent as a spray. Table I shows the limits of detectability for various guanidine compounds when this reagent is used.

TABLE I

Detection limits for various guanidine compounds on paper chromatograms with the diacetyl spot reagent (Smith, 1958)

Compound	μg./cm.² of paper*
Taurocyamine	0.75
Glycocyamine	1.06
Creatine	1.30
Arginine	2.00
Guanidine	1.59
Agmatine	2.00

* Values represent the smallest amount of a compound which was found to give a definite positive test on Whatman 3MM paper.

Hypotaurine was determined either by elution from Dowex 50 (H^+) columns with 2×10^{-3} N HCl (Bergeret and Chatagner, 1954), or by oxidation to taurine with hydrogen peroxide and determination as taurine. In the latter procedure, preliminary removal of endogenous taurine by Dowex 50 (H^+) was necessary.

Inorganic ions were determined as follows: (1) sulfites by the distillation-iodine titration method (Fromageot, Chatagner and Bergeret, 1948); (2) sulfates by the Versene technique (Welcher, 1958); and (3) chlorides by the Mohr technique.

Determination of endogenous levels of various substances was performed on a composite homogenate of 25 worms.

Experimental techniques

In vivo tracer studies were made by injecting worms intracoelomically with labeled metabolites. Leakage of compounds to the environment was found to be negligible during the time periods (12 to 36 hours) involved in these studies. Counting of aliquots of whole homogenates of injected worms showed recoveries of radioactivity which averaged 93.4% of the amount injected (Table II). Self-absorption was found to be negligible if the amount of material on the planchet was kept lower than 200 μg. per square centimeter. Hilchey, Cotty and Henry (1957) arrived at a value of 130 μg. per square centimeter, in similar studies.

The distribution of radioactivity into various compounds was determined in several ways—preparation of radioautographs from paper chromatograms, counting of material eluted from paper chromatograms, cocrystallization of metabolites

TABLE II

Recovery of radioactivity 24 hours after injection

Compound injected	Counts/minute		% recovery
	Injected	Found	
S^{35}-taurine	1.17×10^6	1.04×10^6	89.9
S^{35}-cysteine	8.00×10^4	7.50×10^4	94.0
S^{35}-methionine	2.20×10^7	2.19×10^7	99.5
3-C^{14}-serine	2.20×10^6	1.98×10^6	90.0

from extracts by addition of unlabeled carrier. A Nuclear-Chicago, thin window, gas flow counter was used for all counting.

In vitro studies were made with both fresh homogenates and with the lyophilized enzymatic preparation described previously. Enzyme preparations were dialyzed only when the reaction product of interest was present at a high endogenous level. All samples were prepared and adjusted to proper volume before addition of the enzyme preparation. Incubations usually were for one hour, and were performed at room temperature (22° to 23° C.).

TABLE III

Concentrations of free amino acids and related compounds in Arenicola cristata

Compound	Leucine equivalents/gm. of fresh weight*
Cysteine	3.92 ± 0.21
Homocysteine	0.52 ± 0.10
Aspartic acid	1.05 ± 0.05
Glutamic acid	1.73 ± 0.19
Serine	0.22 ± 0.05
Glycine	17.28 ± 0.09
Arginine	0.50 ± 0.07
Alanine	6.00 ± 0.22
Valine plus methionine	0.55 ± 0.03
Leucine	0.75 ± 0.03
Tyrosine	0.025 ± 0.020
Methionine sulfone	0.32 ± 0.08
Hypotaurine	0.48 ± 0.08**
Cysteinesulfinic acid	0.48 ± 0.05**
Taurine	3.28 ± 0.36**
Taurocyamine	5.06 ± 0.21**

* Values represent equivalent μmoles of leucine, except where otherwise indicated. All values represent mean values of four determinations, with standard errors, on aliquots of a homogenate of 25 worms.

** This value represents actual μmoles rather than leucine equivalents.

THE CHEMICAL COMPOSITION OF ARENICOLA

Vinogradov (1953) has summarized information on the chemical composition of *Arenicola marina* and *A. piscatorum*. These data are assumed to be applicable to *A. cristata*.

During the present study, a few additional determinations of inorganic components were made. Moisture content was determined on two worms. An average of 80.6% H_2O was found. Sulfate, determined on six worms, was 84.1 ± 5.3 μmoles per gram of fresh weight. Chloride was measured on three aliquots of the pooled extract of 25 worms. A level of 254 ± 1.9 μmoles per gram of fresh weight was established. Determinations on four aliquots of the same pooled extract by the colorimetric persulfate method showed total manganese to be less than 3.6×10^{-3} μmoles per gram of fresh weight.

Analyses for amino acids and other related materials were performed on the 25-worm extract. Results are given in Table III. Levels of basic compounds are reported as leucine equivalents. Hypotaurine and acidic compounds are reported directely in micromoles.

The status of cysteic acid as a metabolite is in question. It appears that in most cases where cysteic acid is found in extracts of normal tissues, it represents an artifact created by the extraction and isolation procedures. In the present work with *Arenicola,* it was found that cysteic acid always occurred when extracts were prepared at room temperature and were evaporated on the steam bath. When, however, extraction was carried out in the cold, and evaporation was done *in vacuo* without heat, no cysteic acid was detected. For this reason, cysteic acid is regarded as an artifact in *Arenicola cristata.*

TABLE IV

Qualitative studies in vivo using radioactive compounds

Compound found to be radioactive	Methods by which radioactivity was detected after injection of				
	S^{35}, taurine	S^{35}, methionine	S^{35}, cysteine	S^{35}, cystine	3-C^{14}, serine
Sulfate		1	1	1	
Homocysteine		2, 3			
Cystathionine		2	2	2	2, 4
Cysteine		2, 3	3	2	4
Cysteinesulfinic acid		2, 3, 4	3		
Cysteic acid		2, 3, 4	3		
Hypotaurine		5	5		
Taurine	3, 4	2, 3, 4		3	3

Methods:

1. Fifty mg. nonradioactive Na_2SO_4 added/ml. of sample. Sample acidified with HCl. Sulfate precipitated with slight excess of 10% $BaCl_2$ solution. Precipitate filtered, washed, and ignited for one hour at 1000° C. Ignited precipitate pulverized, distributed on planchet, and counted.

2. Compound extracted from sample by crystallization with nonradioactive carrier. Product recrystallized twice. Crystals dissolved. Solution evaporated on planchet and counted. Purity of final product checked by chromatography in four solvents.

3. Compound isolated by paper chromatography in several solvents. Spots eluted from papers. Eluates evaporated on planchets and counted.

4. Compounds isolated by paper chromatography. Radioautographs prepared from chromatograms.

5. Acidic compounds removed by Dowex 50 (H^+) treatment. Basic fraction oxidized with 30% H_2O_2. Oxidized material again separated with Dowex 50 (H^+). Taurine isolated from acidic fraction by paper chromatography in four solvents. Taurine spots eluted. Eluates evaporated on planchets and counted.

Robin's (1954) report of high arginase activity in homogenates of *A. marina* suggested a need for determination of the endogenous urea level in this worm. Manometric estimations with urease (Umbreit, Burris and Stauffer, 1957) showed, for the 25-worm extract, a urea level of 0.96 ± 0.013 μmoles per gram of fresh weight (four replications). Three standard urea samples were analyzed concurrently. The average value obtained for the standards was 101.6% (range: 97.2% to 105.3%) of the urea added.

Results and Discussion

Qualitative studies

These studies were conducted with the relatively large specimens of *A. cristata* obtained from Woods Hole Marine Biological Laboratory.

S^{35}-taurine was administered to a worm by intracoelomic injection. The worm was dissected after 24 hours, and all organs were shown to contain some radioactivity. Radioautographs prepared from chromatograms, and direct counting of material eluted from chromatograms, revealed no radioactivity in any compound other than taurine. This experiment indicated that taurocyamine, although known to contain sulfur, apparently was not formed from taurine. Therefore, a tracer study of sulfur metabolism in *A. cristata* was undertaken.

Radioactive compounds were given by injection and after 12 to 36 hours the animals were homogenized and extracts made. The results of the tracer studies and the methods used to obtain these results are given in Table IV. The labeled intermediates found did not suggest any qualitative departure from the basic sulfur catabolism scheme known for vertebrates. Taurine was formed from methionine or cystine, presumably via decarboxylation of cysteinesulfinic acid to hypotaurine with subsequent oxidation of the latter. Cysteine and cystine were metabolically interconvertible, although the rate and nature of this conversion are not known. A cystathionine pathway from methionine to cysteine exists and, as is also known in the vertebrate, involves serine. In no case was any radioactivity detected in taurocyamine, even 36 hours after injection of cystine.

Quantitative studies

These studies were conducted with the small specimens of *A. cristata* obtained from the St. Petersburg, Florida, area. In all studies using labeled compounds, total recovery of radioactivity was assumed. With this assumption, data regarding distribution of radioactivity may be regarded as representing the distribution of the entire metabolic pool of the injected compound. Because of the complete overlap between elution peaks from Dowex 50 (H^+) columns for sulfate and for cysteic acid, radioactivity levels for these compounds are pooled in all values given for distribution of radioactivity after injection of S^{35}-labeled compounds.

Tracer studies showed that *A. cristata* converted approximately 50% of injected S^{35}-cysteine into oxidized, strongly acidic entities within 12 hours. With cystine, the process was much slower, only about 18% being oxidized in 12 hours. The 50% level was attained with cystine by 36 hours.

When 3-C^{14}-serine was injected into the worms, almost 60% of the total radioactivity appeared within 24 hours in strongly acidic compounds, *i.e.*, those which passed through the Dowex 50 (H^+) column. Most of these components showed no reaction with ninhydrin. An estimated 12% of the total radioactivity was associated with strongly acidic sulfur-containing compounds (Table V). This was about the same amount of incorporation as was obtained in 12 hours with S^{35}-cysteine, excluding inorganic sulfate.

With methionine, only 17.8% of the total radioactivity appeared in the acidic fraction in 24 hours. Of this, 13.5% was in sulfate plus cysteic acid, 2.7% in taurine plus an unidentified compound, and 1.6% in cysteinesulfinic acid. Pre-

sumably, the rate of demethylation of methionine to form homocysteine was the limiting factor in this system.

Relatively high percentages of radioactivity were incorporated into sulfate, as compared with other sulfur compounds, after injection of S^{35}-cysteine or

TABLE V

Distribution of radioactivity among acidic compounds in Arenicola cristata following injections of labeled compounds

Injected compound	Fraction	Counts/minute/gm. of fresh weight	Distribution of total radioactivity %
S^{35}-cysteine (extraction 12 hours after injection)	Whole extract	21,360	100
	Acidic portion	11,070	51.83
	Sulfate + cysteic acid	8,460	39.61
	Taurine	490	2.29
	Cysteinesulfinic acid	1,208	5.66
	Compounds X_1 and X_2	912	4.27
S^{35}-cystine (extraction 12 hours after injection)	Whole extract	23,925	100
	Acidic portion	4,250	17.77
	Sulfate + cysteic acid	3,938	16.46
	Taurine	155	0.65
	Cysteinesulfinic acid	79	0.33
	Compounds X_1 and X_2	78	0.33
S^{35}-cystine (extraction 36 hours after injection)	Whole extract	21,510	100
	Acidic portion	10,823	50.32
	Sulfate + cysteic acid	8,708	40.48
	Taurine	1,462	6.80
	Remainder	653	3.04
S^{35}-methionine (extraction 24 hours after injection)	Whole extract	438,000	100
	Acidic portion	78,000	17.8
	Sulfate + cysteic acid	59,300	13.5
	Taurine + X_1	11,500	2.7
	Cysteinesulfinic acid	7,200	1.6
3-C^{14}-serine (extraction 24 hours after injection)	Whole extract	14,710	100
	Acidic portion	8,600	58.5
	Taurine*	1,860	12.6
	Compounds not containing sulfur	6,740	45.9

* Since many highly acidic carbon compounds emerge at the front during elution of the Dowex 50 (H^+) columns, radioactivity associated with cysteic acid cannot be isolated. Cysteinesulfinic acid shows no radioactivity. Therefore, it is assumed that cysteic acid also is not radioactive and that all radioactivity appearing in sulfur-containing strongly acidic compounds is associated with taurine.

S^{35}-cystine (Table V, Fig. 2). These percentages are of the same order as those reported from other sources (Kay and Entenman, 1959; Skarzynski, Szczepkowski and Weber, 1959; Eldjarn, Pihl and Svedrup, 1956; Tarver and Schmidt, 1942).

Fromageot (1953), Kearney and Singer (1953a, 1953b), Chatagner *et al.* (1952) and others have all emphasized that the most important pathway of sulfur

from cysteine to sulfate is through transamination between cysteinesulfinic acid and α-ketoglutaric acid to form glutamic acid and an unstable intermediate, β-sulfinylpyruvic acid. The latter decomposes into sulfite, which is oxidized to sulfate, and into pyruvic acid, which by transamination with glutamic acid regenerates α-ketoglutaric acid and forms alanine.

It has been demonstrated that both enzymatic and non-enzymatic oxidation of sulfite to sulfate occur in mammals (Heimberg, Fridovich and Handler, 1953; Fridovich and Handler, 1956). The enzymatic process is heat-labile. When sodium sulfite solutions were incubated with the soluble enzyme preparations from *A. cristata,* the results in terms of sulfite remaining after one hour, although somewhat erratic, indicated that no decrease in activity was effected by boiling the enzyme preparation. Thus, the enzymatic mechanism may be of little importance in *Arenicola,* or may be absent.

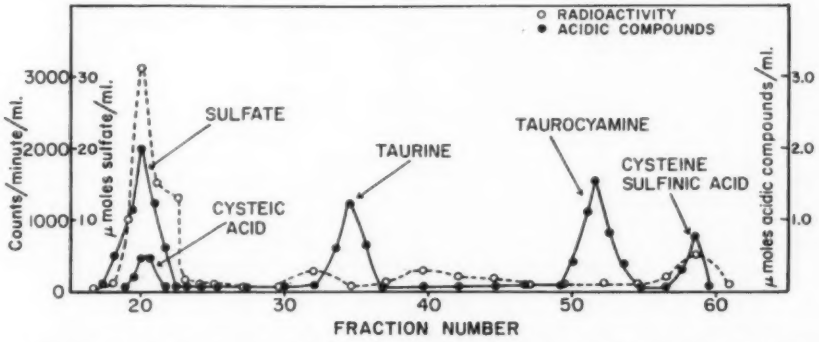

FIGURE 2. Acidic components from 1 gm. of *Arenicola cristata* 12 hours after injection of S^{35}-cysteine. Fractions are 1-ml. portions of eluate from 60 × 0.9 cm. Dowex 50 (H^+) column.

The activity of the cysteinesulfinic acid-α-ketoglutaric acid transaminase system *in vitro* may be evaluated from Table VI, which presents the most consistent series of experimental results obtained in several trials of this system. The values reported for glutamic acid represent increases above the endogenous level for this substance. There seems little reason to doubt the formation of sulfate via the transaminase-β-sulfinylpyruvate pathway.

The formation of hypotaurine in the mammal by decarboxylation of cysteinesulfinic acid has been established for some time (Bergeret and Chatagner, 1952; Bergeret, Chatagner and Fromageot, 1952; Awapara and Wingo, 1953). Pyridoxal phosphate has been shown to act as coenzyme for this reaction and to resist dialysis, indicating that it is tightly bound to the apoenzyme (Bergeret and Chatagner, 1952; Blaschko and Hope, 1954; Hope, 1955). In the present study, hypotaurine was found to occur in *A. cristata* at an endogenous level of 0.5 μmole per gram of fresh weight. Attempts to show decarboxylation of cysteine-sulfinic acid *in vitro* gave highly variable results. Whole homogenates and solutions of the lyophilized soluble fraction of the worm were tested. Pyridoxal phosphate was added to

samples at levels of 4 to 8 μg. per milliliter. In all manometric experiments carbon dioxide was released. Chromatography of reaction mixtures after 60-minute incubation periods revealed distinctly more hypotaurine in reactive samples than in controls, based on comparison of colors and sizes of spots on ninhydrin-developed papers. Colorimetric estimation showed that an erratic increase in hypotaurine occurred in samples as compared with controls. The reaction was heat-labile.

TABLE VI

*Cysteinesulfinic acid-α-ketoglutamic acid transamination**

Sample number	ml. of extract added	μmoles of cysteinesulfinic acid added	μmoles of α-ketoglutaric acid added	μmoles of glutamic acid formed/gm. of fresh weight/hour
1	1.0**	25	25	0.0
2	1.0**	25	25	0.0
3	0.0	25	25	0.0
4	0.0	25	25	0.0
5	1.0	25	0	0.0
6	1.0	25	0	0.0
7	1.0	0	25	0.0
8	1.0	0	25	0.0
9	1.0	25	25	12.1
10	1.0	25	25	6.0
11	1.0	25	25	7.2
12	1.0	50	25	10.5
13	1.0	50	25	9.9
14	1.0	50	25	10.5
15	1.0	75	25	11.0
16	1.0	75	25	11.0
17	1.0	75	25	11.6
18	1.0	100	25	11.0
19	1.0	100	25	10.5
20	1.0	25	50	8.3
21	1.0	25	50	8.3
22	1.0	25	75	9.9
23	1.0	25	75	9.3
24	1.0	25	100	9.3
25	1.0	25	100	9.9
26	1.0	50	50	12.7
27	1.0	50	50	12.7
28	1.0	100	100	15.0
29	1.0	100	100	17.7
30	2.0	25	25	15.0
31	2.0	25	25	12.2
32	2.0	25	25	12.7
33	3.0	25	25	19.4
34	3.0	25	25	17.2
35	3.0	25	25	17.2

* Conditions: All solutions prepared in 0.01 *M* phosphate buffer, pH 7.3. Total volume: 5.0 ml. Each ml. of extract contained 50 mg. of lyophilized soluble fraction of *A. cristata*. Aerobic incubation of samples for 120 minutes at temperatures of 22° to 23° C. Acids neutralized with NaOH prior to making stock solutions.

** Extract boiled for 5 minutes before addition to sample.

TABLE VII

*Oxidation of hypotaurine to taurine**

Sample number	ml. of homogenate added	Molarity of hypotaurine	μmoles of taurine formed/hour/gm. of fresh weight
1	1.0	0.0040	12.0
2	1.0	0.0040	12.5
3	1.0	0.0040	13.0
4	1.0	0.0035	11.5
5	1.0	0.0035	11.5
6	1.0	0.0035	12.0
7	1.0	0.0030	10.0
8	1.0	0.0030	11.5
9	1.0	0.0030	11.0
10	1.0	0.0025	8.5
11	1.0	0.0025	10.5
12	1.0	0.0025	9.0
13	1.0**	0.0025	8.0
14	1.0**	0.0025	8.5
15	2.0	0.0025	9.0
16	3.0	0.0025	9.0
17	0.0	0.0025	0.0

* Conditions: All solutions prepared in 0.01 *M* phosphate buffer, pH 7.3. Total volume: 11.0 ml. Fresh homogenate of *A. cristata*: 1 ml. equivalent to 200 mg. of fresh weight. Aerobic incubation of samples for 60 minutes at temperatures of 22° to 23° C. Hypotaurine neutralized with NaOH before stock solution was prepared. Taurine determined by separation on Dowex 50 (H^+), columns followed by ninhydrin treatment of aliquots of filtrates.

** Homogenates boiled for 5 minutes before addition to sample.

The oxidation of hypotaurine to taurine was studied with fresh whole homogenates and with the soluble enzyme extract. The reaction appeared to be non-enzymatic; it was not hindered by prolonged boiling (up to 20 minutes) of homogenates and extracts. Table VII shows the most consistent results obtained from the series of experiments on this reaction. Values for taurine formation are based on increases above the endogenous level. A reciprocal plot of this experiment was derived by least squares analysis. Statistical testing indicates that the line does not pass through the origin ($0.02 > p > 0.01$). Other less extensive experiments gave regression lines which were not significantly different from lines passing through the origin. It is postulated that the reaction is non-enzymatic because of its total insensitivity to prolonged heating. Metal ion catalysis may be involved here since the presence of the worm homogenate or extract is necessary to the reaction. It is further suggested that the reciprocal plot should not pass through the origin, as there is undoubtedly some substrate level at which catalyst saturation occurs and the reaction becomes zero order.

Repeated attempts were made to demonstrate transamidination between arginine and a variety of acceptor compounds—taurine, hypotaurine, cysteine-sulfinic acid, cysteic acid, cysteine, cystathionine, and mercaptoethylamine. All results were negative. Care was taken to add sufficient arginine (20 or more μmoles per milliliter of sample) so that arginase activity could not seriously deplete the supply of the amidine group donor during incubation.

The experiment with the S^{35}-taurine injection was repeated with taurine of higher specific activity. Twelve hours after injection, the animal was homogenized and the homogenate was fractionated by chromatography on the Dowex 50 (H^+) column. When the fractions were checked for radioactivity, a slight amount of radioactive sulfur was detected in the taurocyamine. On the assumption of no leakage of compounds to the environment, and with the endogenous concentration of taurine known, it was calculated that only 0.60 μmole of taurocyamine—0.10 μmole per gram of fresh weight—had been formed in 12 hours. The correlation of taurocyamine content with radioactivity in 1-ml. fractions eluted from the column is shown in Figure 3.

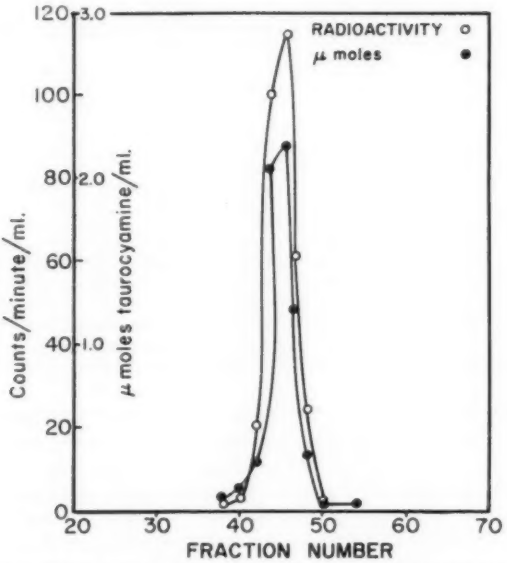

FIGURE 3. Taurocyamine fractions from 1 gm. of *Arenicola cristata* 12 hours after injection with S^{35}-taurine. Fractions are 1-ml. portions of eluate from 60 × 0.9 cm. Dowex 50 (H^+) column.

As soon as this information was obtained, a solution of moderately radioactive S^{35}-taurine was prepared for use in *in vitro* studies. Three experiments—two with fresh homogenates of *A. cristata* and one with a lyophilized soluble fraction—were performed in an attempt to obtain transamidination between arginine and taurine. Success was achieved with only one sample, which was part of the experiment using the lyophilized preparation. After incubation for 60 minutes, 0.004 μmole of taurocyamine was formed by this sample, which contained 100 μmoles of arginine, 5 μmoles of taurine (representing 2335 counts per minute), and an amount of extract equivalent to 0.61 gm. of fresh weight of worm tissue. Total volume was 5.0 ml.

From the *in vivo* results, it might be assumed that the slow rate of tauro-

cyamine formation resulted from competition between arginase (Robin, 1954) and transamidinase for the limited arginine supply. The over-all endogenous level of arginine was about 0.5 μmole per gram of fresh weight, all of which seemed to occur in the wall of the digestive tract. However, the assumption of competition was not supported by *in vitro* results, as sufficient arginine was present to supply both systems adequately. Dr. James B. Walker (personal communication) has speculated that his transamidinase preparations from mammalian tissues and from *Streptomyces griseus* probably would show similar low levels of activity toward almost any suitable amidine group acceptor.

Summary and Conclusions

The pattern of sulfur metabolism in *Arenicola cristata* was examined. Qualitatively, it differs little from the pathway known in mammals, except in one of the terminal reactions. This reaction leads to incorporation of cysteine sulfur and, hence, of methionine sulfur into the compound taurocyamine by transamidination between taurine and arginine, as suggested by Robin (1954).

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HISTOLOGICAL STUDIES ON THE DIGESTIVE SYSTEM OF A STARFISH, HENRICIA, WITH NOTES ON TIEDEMANN'S POUCHES IN STARFISHES[1]

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In starfishes of the genus *Henricia* (Family Echinasteridae), the digestive tract is a complex system presenting many highly specialized features and contrasting markedly with the much simpler digestive organs of forms such as *Asterias* or *Pisaster*. In consequence of a number of descriptive and experimental studies, structural and functional aspects of feeding and digestion in Asteriidae are reasonably well understood; in contrast, very little is known of the details of structure and function in the alimentary tract of *Henricia*. From the functional standpoint, for instance, we remain in ignorance of the normal food of *Henricia* (Mortensen, 1927, p. 120), and of its mode of feeding. Anatomy and histology have received somewhat more attention: Cuénot (1887) devotes some descriptive remarks to the digestive system in *Cribella oculata* (= *Henricia sanguinolenta*), and Hayashi (1935) includes sections on the structure and histology of the digestive tract in his article on the anatomy of a Japanese variety of *H. sanguinolenta* which later (1940) he considers a separate species, *H. ohshimai*. Hayashi's account is reasonably accurate as far as it goes, but in terms of the digestive tract it is disappointingly superficial, omitting to mention several conspicuous features and relationships that are both interesting and significant. The present study was undertaken originally as a necessary foundation for contemplated experimental studies on the digestive tract. It presents a more nearly complete description of anatomical and histological details, seeks to combine these with histochemical characteristics of parts of the digestive system as possible clues to function, and draws attention to unique features of the system unaccountably omitted from previous descriptions.

Prominent among these are the structures associated with the pyloric caeca which Ludwig and Hamann (1899) named "Tiedemann's pouches" in recognition of their discoverer. In his epochal monograph of 1816, which laid the foundations of our modern knowledge of echinoderm morphology, Friedrich Tiedemann briefly described a detail of structure in *Astropecten auranciacus*, as follows (p. 48): "On the lower surface of each caecum there is a small, elongate, and hollow appendage, which commonly contains a yellowish fluid. Perhaps this appendage is a kind of secretory organ." Cuénot (1887) called attention to the singular development of these structures in starfishes of the families Asterinidae and Echinasteridae (p. 34): ". . . the median sac is considerably elongated and forms a voluminous reservoir, which, in the natural position of the animal, is situated below the radial caecum; this reservoir runs about half or three-quarters the

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length of the caecum and is marked by regularly spaced oblique folds; it opens widely into the gastric sac, of which it is nothing more than a continuation." (See also Cuénot's Plate II, Figures 11 and 18.)

Considering the conspicuous and distinctive nature of these structures in the starfishes that possess them, it is surprising that Tiedemann's pouches (or Tiedemann's diverticula, as they are also called) have attracted so little attention with regard to either structural details or functional possibilities. Vogt and Yung (1888), referring to the relatively small pouches in *Astropecten*, mention that solid food has been observed in the pouches but not in the pyloric caeca themselves. Irving (1924), in his discussion of ciliary currents in *Patiria miniata*, describes Tiedemann's pouches as the median ducts of the caeca which bear the glandular pockets on their aboral walls. Misled by this description, I later (Anderson, 1953) used the same terminology in discussing the pyloric caeca of *Asterias forbesi*. It must be noted that the caeca of the family Asteriidae do not possess Tiedemann's pouches and that where they do occur, these pouches are not the median ducts themselves but form extensive appendages on the oral sides of the median ducts. Finally, Hayashi (1935) pays practically no attention to these conspicuous structures in his anatomical study of *Henricia*, saying only (p. 9), "Each caecum has a spacious median canal which is elongated dorso-ventrally in cross-section, and the wall is thin and folded."

We thus appear to lack altogether any detailed information on structure and function in Tiedemann's pouches. Accordingly, in the present study some emphasis is placed upon the anatomical and histological features of Tiedemann's pouches in *Henricia*, and upon the very considerable differences that exist between the pouches in *Henricia* and those in *Patiria*, a member of the other family (Asterinidae) in which such pouches typically occur, according to Cuénot (1887). Where pertinent, brief remarks are included concerning preliminary studies of the pouches in *Asterina gibbosa*, *Astropecten irregularis* and *armata*, and *Linckia guildingi*.

These studies were begun during tenure of a John Simon Guggenheim Memorial Fellowship in 1958–59, at the Hopkins Marine Station of Stanford University, Pacific Grove, Calif., and continued at the Marine Biological Laboratory, Woods Hole, Mass. The generous support of the Guggenheim Memorial Foundation, and the hospitality and cooperation of the Director and Staff of the Hopkins Marine Station, are hereby gratefully acknowledged. I also acknowledge with thanks the aid of the following, who provided specimens of starfishes from the indicated areas: Dr. F. S. Russell, Plymouth; Dr. R. A. Boolootian, Los Angeles; Aage Möller Christensen, Helsingör; and Jonathan Green, Hawaii.

Materials and Methods

Small to moderate-sized specimens of *Henricia leviuscula* were collected beneath stones in the intertidal zone at Point Piños, Pacific Grove, Calif. They were maintained in running sea-water in one-gallon jars provided with escape-proof collars of plastic screening. The specimens could not be induced to feed upon any of the variety of items readily accepted by *Patiria* and other species, but as they remained in apparently vigorous condition for periods of two months or more,

the possibility exists that they were feeding unobserved upon suspended particulate material in the water, or upon the algal and bacterial film on the walls of their containers. Comparative and confirmatory observations were made at Woods Hole on locally-collected specimens of *Henricia sanguinolenta* maintained under similar conditions. It is apparent that the two species do not differ significantly in the details of their internal anatomy, and the results to be reported are composite.

Gross anatomical studies involved specimens dissected after treatment with $MgCl_2$ (8% in tap water) to prevent movement or autotomy. For histological examination, tissues were fixed in Helly's fluid, washed, dehydrated, imbedded in paraffin, and sectioned serially at 7 to 10 μ; or fixed in Baker's formol-saline, post-chromed 24 hours in potassium bichromate, imbedded in gelatin, and sectioned on the freezing microtome. Tissues that included parts of the body wall were decalcified by soaking for approximately a week in 5% aqueous disodium ethylene-dinitrilo-tetraacetate (EDTA), a chelating agent, between fixation and dehydration steps.

For general study and orientation, paraffin sections were stained with Harris' hematoxylin and eosin. For the demonstration of muscle fibers, connective tissue, cell membranes, flagella and their basal bodies, and secretion granules, excellent results were obtained by the use of Mallory's phosphotungstic acid hematoxylin. Glycogen and other polysaccharide compounds were demonstrated by a periodic-acid-Schiff technic, controlled by salivary digestion, and metachromatic substances were stained by overnight exposure to very dilute solutions of toluidine blue. Steedman's Alcian Blue technic, as given by Pearse (1953), was employed for staining acid mucopolysaccharides. The frozen sections of gelatin-imbedded material were colored with Sudan black, counterstained with carmalum, to reveal lipid deposits.

Observations

A. Anatomy

As in starfishes generally, the digestive tract of *Henricia* is essentially a tube, running from mouth to anus in the short vertical axis of the body, divided into specialized successive regions termed cardiac stomach, pyloric stomach, and intestine (esophagus and rectum are additionally distinguished by many authors). The pyloric stomach gives off a pair of glandular appendages, the pyloric caeca, into each ray, and the intestine (or rectum) bears an exceptionally well-developed group of sacculate appendages, of unknown function, termed rectal caeca or intestinal caeca.

Viewed from below, as in Figure 1, the mouth appears as a stellate opening bounded by 5 peristomial lobes which approach each other centrally. When the animal is undisturbed, the mouth is usually partially open, as in this photograph. Hayashi (1935) terms the cardiac stomach, into which the mouth opens, "rudimental." It is relatively small, compared with that of *Asterias*, for example, and lies wholly within the circular frame bounded by the proximal ambulacral ossicles (Fig. 2). The stomach consists largely of a series of structures termed "esophageal pouches" by Cuénot (1887). There are 10 of these, 5 radial and 5 interradial in position; each radial pouch lies just medial to the large proximal ambulacral ossicle in its ray and is movably bound to this ossicle by a pair of

Figures 1 through 27 illustrate features of the digestive system in *Henricia;* Figures 28 through 31 show details of Tiedemann's pouches in *Patiria miniata.*

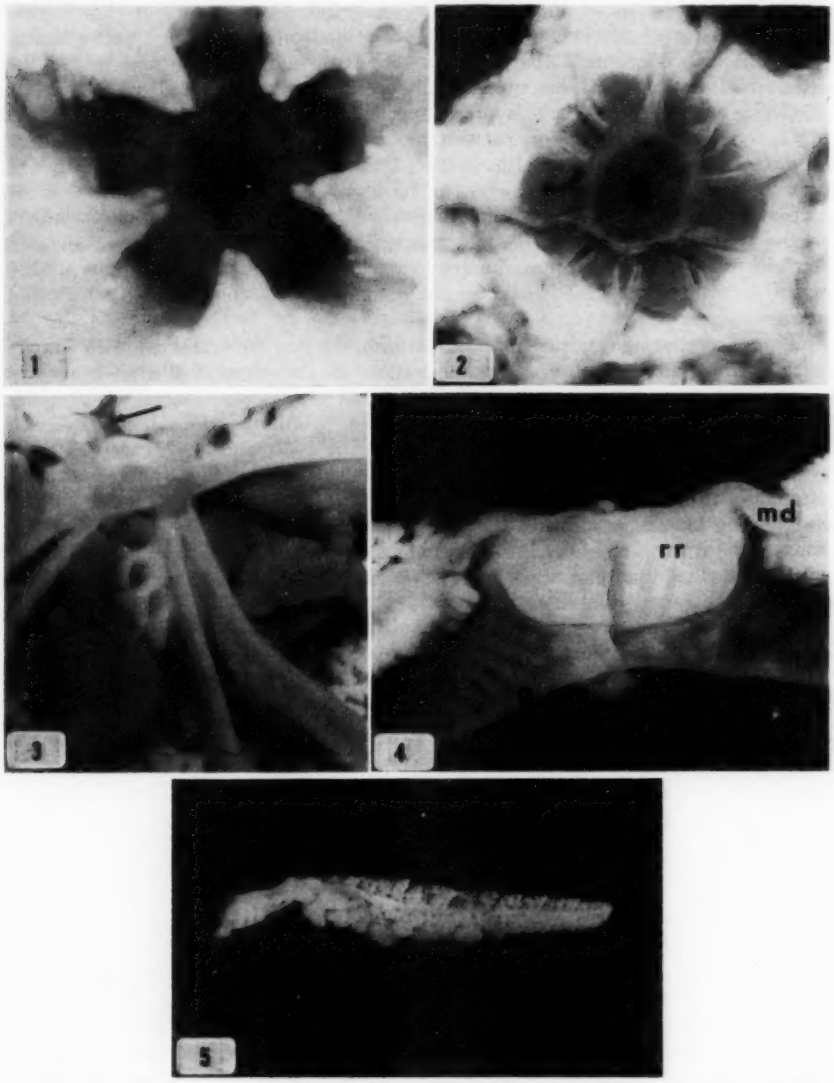


FIGURE 1. Oral view of living specimen, showing partially open mouth surrounded by peristomial membrane. Approximately 20 ×.

FIGURE 2. Cardiac stomach from above, after removal of all aboral parts by transecting the pyloric stomach at the line indicated by the arrow. Note radial pouches (*r*); interradial

stout, fibrous retractor strands. From the same origins a pair of longer strands pass upward to insertions higher on the wall of the stomach. Hayashi remarks on the thick and compact appearance of these retractor strands in contrast to the highly branched fibers in *Asterias;* in *Henricia,* although short strands do extend to the outer surface of the adjacent esophageal pouches, these are neither so numerous nor so extensive as those forming what has been termed the intrinsic retractor mechanism in *Asterias* and *Patiria* (Anderson, 1954, 1959).

Above the interradial pouches the wall of the stomach folds inward longitudinally to form 5 or 6 (usually 5) large, centrally-directed bulges that almost occlude the lumen of the stomach but leave a narrow, stellate central passageway (Fig. 2). These swollen vesicles are separated from one another by deep, radiating folds which are restricted lateral extensions from the angles of the central passage and which, in consequence of their radial positions, form conspicuous gutters leading upward from the cavities of the radial esophageal pouches. Above the level of these alternating vesicles and gutters the stomach is encircled by a slender fibrous girdle, into which the longer retractor strands from each ray insert. There is no real constriction marking the separation between cardiac and pyloric portions of the stomach, and in the absence of other landmarks the regions above this circumferential girdle will be considered as belonging to the pyloric stomach. This is in agreement with the situation in *Patiria,* where a similar fibrous girdle has been taken to mark the upper limit of the cardiac stomach (Anderson, 1959).

Just above the level of the girdle, the pyloric stomach tapers somewhat, and its walls are perforated by a marginal opening at each of its radial angles. Each of these 5 openings leads into a duct which immediately bifurcates, giving rise to pale, translucent, cylindrical branches forming the tubular proximal channels that expand vertically into the two Tiedemann's pouches in its ray (Figs. 3, 4, 5). It should be noted that each marginal passage constitutes, in effect, a continuation of one of the radial gutters leading upward between the vesicles of the cardiac stomach, from a radial esophageal pouch.

Above the marginal openings, the pyloric stomach forms 10 radiating branches, rather deep in the vertical dimension, two leading towards each ray. In each of these branches the side walls evaginate and are thrown into coarse folds, forming what I shall call radial reservoirs, with creamy-white, opaque walls. The radial reservoir tapers upward and outward, narrowing as the underlying Tiedemann's pouch expands, and becomes the median duct of one of the paired pyloric caeca

pouches (*i*); vesicles (*v*) surrounding the central aperture; and the stout retractor harness sending strands over the radial pouches to attach on the circumferential girdle (*arrow*) marking the boundary between cardiac and pyloric stomach. Approximately 15 ×.

FIGURE 3. Aboral parts of the digestive system viewed from below. Arrow indicates the central lumen of the pyloric stomach, from which the caecum-pouch complexes radiate in pairs (for details of these parts, see following figures). Note the well-developed rectal caeca (*rc*). Approximately 15 ×.

FIGURE 4. Basal parts of one pair of caecum-pouch complexes, cut off from central attachments and spread apart to show components. Tiedemann's duct widens into Tiedemann's pouch (*Tp*), with gutter (*g*) forming its oral margin; above, the median duct (*md*) of the caecum widens into the thick-walled radial reservoir (*rr*) by which it connects to the pyloric stomach. Approximately 15 ×.

FIGURE 5. A single caecum-pouch complex, cut off at left from its attachment to the pyloric stomach, showing relationship between Tiedemann's pouch (note parallel channels traversing its walls), radial reservoir, median duct, and lateral diverticula. Approximately 5 ×.

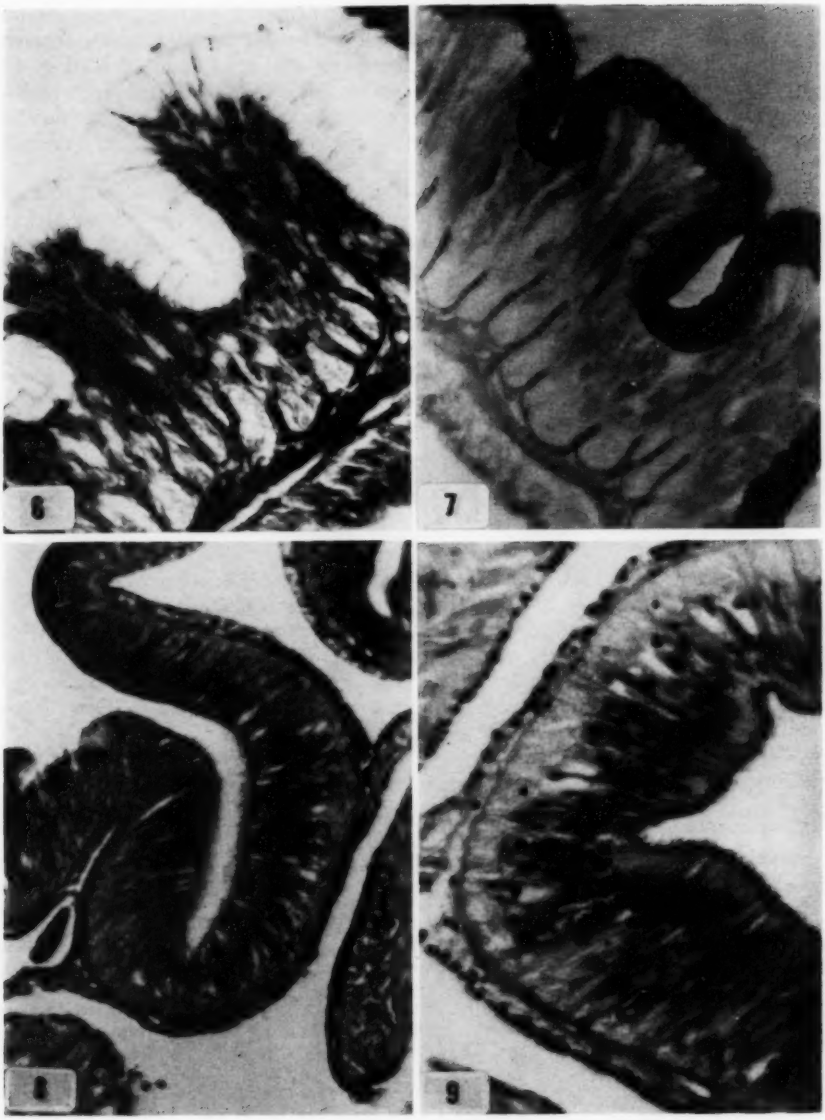


FIGURE 6. Extreme lower end of cardiac stomach ("esophagus"); note the heavy partitions extending upward from the connective-tissue layer; the tall epithelial cells with apparently empty distal ends; and the strands of secretory granules marking locations of the few zymogen cells in this area. Phosphotungstic acid hematoxylin; 470 ×.

(Figs. 4, 5). The first of the lateral diverticula of the pyloric caecum leaves the median duct somewhat beyond the end of the radial reservoir.

The figures clearly demonstrate that the pyloric caecum-Tiedemann pouch complex is dual in nature. The aboral part, consisting of the median duct with its many lateral diverticula, has its affinities with the radial reservoir, of which it appears to be a continuation. Tiedemann's pouch, on the other hand, altogether different in gross appearance, originates almost at the junction between cardiac and pyloric portions of the stomach and is evidently closely related to the lower region. The two ducts, the median duct and what we may call Tiedemann's duct, accompany each other and are closely bound together, but their cavities are completely separated by a continuous partition. Passing outward, the lower duct becomes a gutter that forms the oral margin of the deep, narrow pouch, and the body of the pouch itself extends between this gutter and the median duct of the pyloric caecum above. Throughout its length, the side walls of the pouch are traversed by a series of diagonal, parallel lines, giving the external appearance of the "regularly spaced oblique folds" remarked by Cuénot (1887). Proceeding outward, the depth of the pouch decreases gradually, and at its outer end (about two-thirds the length of the caecum from its base) the oral gutter enters the floor of the median duct of the caecum (Fig. 5).

The roof of the pyloric stomach shows folds corresponding to the radial branches of the walls; these converge centrally upon the opening of the intestine. I can add nothing to Hayashi's description of the anatomy of this portion of the digestive tract, or of the rectal caeca which are extraordinarily well developed in *Henricia* (Fig. 3).

B. Histology

Histologically, the gut wall of *Henricia* presents the general features that appear to be standard throughout the Asteroidea. The usual tissue layers are present—peritoneum, muscular layers, connective-tissue layer, nerve plexus layer, and lining epithelium—and occupy the same relative positions as in other starfishes. The chief inadequacies of Hayashi's otherwise good account describing these layers involve his omission of significant details concerning the lining epithelium. This is basically a very tall layer, composed of what may be termed "typical" cells—long, slender cells, crowded together, with their ovoid nuclei lying at varying levels in the epithelium; each cell is provided, usually, with a single flagellum, springing from an apical basal body that sends a tapering fibril downward towards the nucleus. Better developed in some parts of the stomach than in others, longitudinal supporting fibrils also run through the basal portions of the

FIGURE 7. Adjacent section, showing intense metachromatic staining with toluidine blue in the distal areas that do not stain with PTAH. The faint coarse granules scattered in the epithelial cells are also metachromatic, as are the contents of the mucous goblets. 470 ×.

FIGURE 8. Section across a radial pouch, low in the cardiac stomach, showing tall, flagellated epithelium and relative scarcity of secretory cells in the pouch itself. Note also the relationship of the retractor strands to the connective-tissue layer in this region. Periodic-acid-Schiff, Weigert hematoxylin, fast green; 235 ×.

FIGURE 9. Detail of epithelium in a radial pouch. Note the tall, crowded, flagellated cells with their conspicuous brush border, and the well-developed nerve plexus layer through which bases of the epithelial cells extend to attach on the connective-tissue layer. Alcian Blue-carmalum; 470 ×.

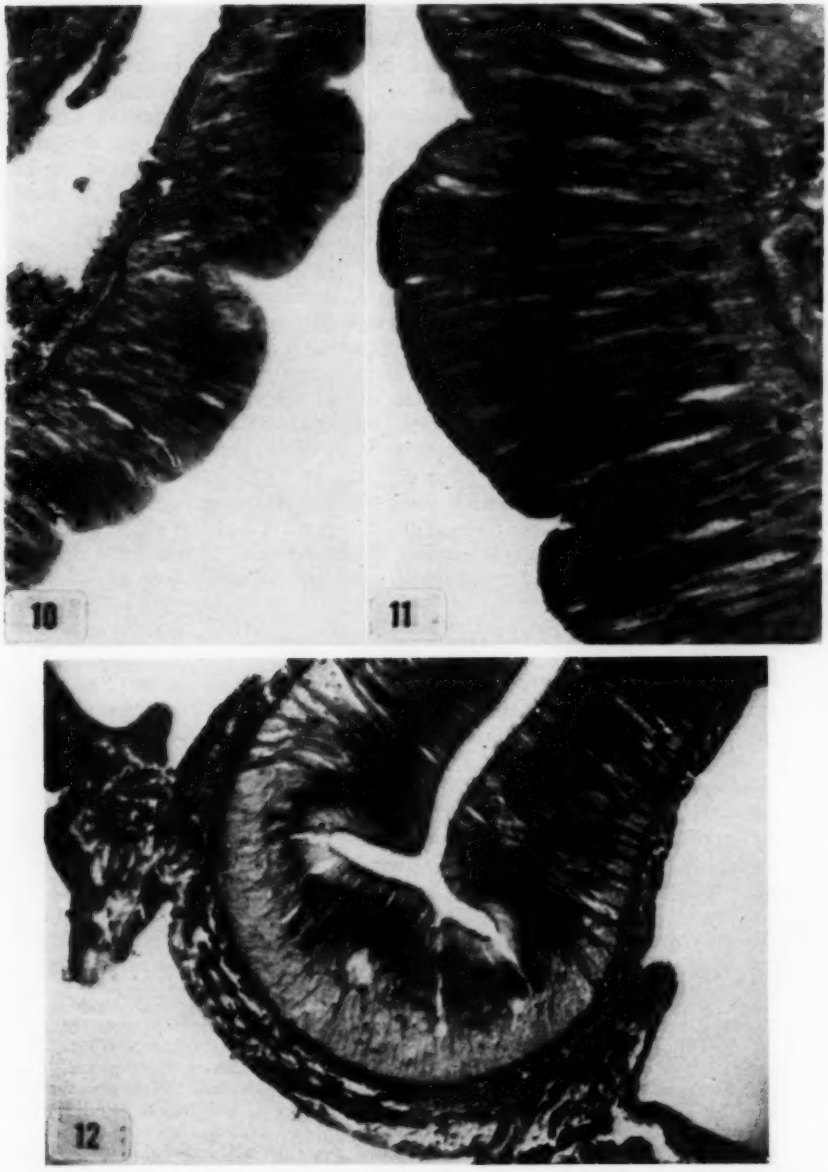


FIGURE 10. An interradial pouch area, showing abundance of zymogen cells; note also the scanty nature of brush border development and flagella. PTAH; 235 ×.

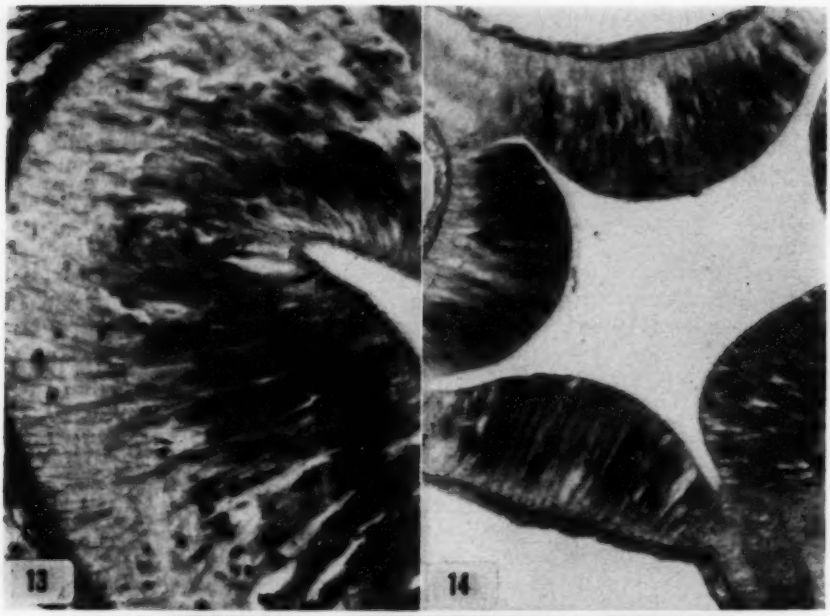
cells and enter the roots that attach to the surface of the underlying connective-tissue layer. The apical ends of typical cells are provided with a brush border, sometimes rather scanty, about the base of the flagellum. Variations in such typical cells, and in the distribution of different types of secretory cells among them, form the basis of the marked regional specializations characteristic of the digestive tract in *Henricia*.

At the extreme oral end of the cardiac stomach, in the region termed "esophagus" by Hayashi and others, the epithelium lies in numerous folds, supported by lamellar inward extensions from the basal connective-tissue layer. Scattered among the typical epithelial cells here are occasional clumps or strings of secretory granules, and somewhat more numerous tall, flask-shaped mucous goblets with distorted, deeply staining basal nuclei. These features are brought out by staining with phosphotungstic acid hematoxylin (PTAH) and are illustrated in Figure 6. By far the most conspicuous aspect of the epithelium in this region, however, concerns the clear and empty appearance of the apical ends in all of the otherwise typical cells here; PTAH stains nothing (other than flagellary basal bodies) in the distal quarter or so of these cells. In contrast (Fig. 7), treatment of adjacent sections with dilute toluidine blue elicits a most vivid gamma metachromasia in precisely those parts of the cells that fail to stain with PTAH. The same regions are stained by Harris' hematoxylin, selectively colored by Alcian Blue, and even after salivary digestion give a positive reaction with the periodic-acid-Schiff technic. Such staining behavior indicates that the apical portions of the typical cells in this portion of the stomach are filled with masses of an acid mucopolysaccharide, and this is the only place in the entire digestive tract where such materials are found to be so intimately and so copiously associated with "typical" epithelial cells. Metachromatic staining, along with the other reactions described for these cells, is also exhibited by the globular or flocculent contents of the mucous goblets, by material associated with the brush border, and by numerous rather coarse granules or deposits randomly scattered in the deeper parts of the epithelial cells (Fig. 7).

Above this region the metachromatic staining of the typical cells gradually disappears, except for that associated with the brush border, and the wall of the stomach differentiates into its radial and interradial pouches. A clear distinction can usually be made between these two series of pouches on the basis of their epithelial characteristics. In the relatively narrow and deep radial pouches, the epithelium consists almost entirely of typical cells, very crowded, with conspicuous, long flagella and well-developed apical brush borders (Figs. 8, 9); in these areas there are few secretory cells, but some zymogen cells are present and some mucous cells containing flocculent secretion. By contrast, the larger and broader interradial pouch areas exhibit an epithelium very rich in mucous goblets and with relatively large numbers of zymogen cells (Figs. 10, 11). Although the typical

FIGURE 11. Mucous gland cells in an interradial pouch area comparable with that shown in Figure 10. Periodic-acid-Schiff after salivary digestion, Weigert hematoxylin, fast green. Note the tall, slender, deeply-staining mucous goblets, and the diffuse positive stain in the distal parts of the epithelium after removal of all glycogen. 470 ×.

FIGURE 12. Radial gutter in cardiac stomach, upward extension of a radial pouch. Tall, crowded, conspicuously-flagellated cells, well-developed nerve plexus layer; note also the large, darkly-stained mucous goblet. PTAH; 235 ×.



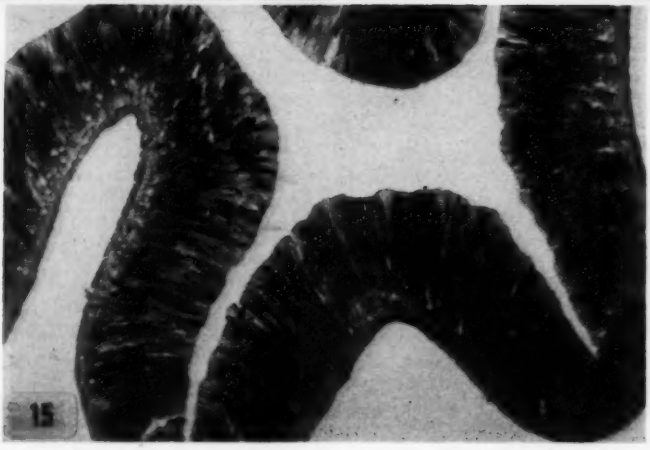


FIGURE 13. Detail of a region similar to that shown in Figure 12. Note bulbous mucous goblets, with scattered spindle-shaped bodies deep in the epithelium (*cf.* Fig. 16). PTAH; 470 ×.

FIGURE 14. Frontal section through the central aperture leading into the pyloric stomach, showing secretory type of epithelium with numerous mucous glands, scanty brush borders and flagella. PAS-Weigert-fast green, salivary digestion; 235 ×.

FIGURE 15. Frontal section through portion of radial reservoir of the pyloric stomach, stained to reveal the abundance of zymogen cells in this region. PTAH; 235 ×.

cells that are found here show flagella on close examination, these are sparse and relatively short, and the brush borders are scanty. These general distinctions in epithelial characteristics follow consistently upward through the cardiac stomach; the crowded, flagellated, conspicuously brush-bordered cells of the radial pouches continue upward through the radial gutters, crossing into the pyloric stomach as tracts leading to the marginal openings in its walls, with the addition of a large, dense, bulbous type of mucous gland cell (Figs. 12, 13). The richly secretory epithelium of the interradial pouch areas, with its short, scanty flagella and brush borders, extends to clothe the centrally-directed vesicles of the cardiac stomach and even to furnish the characteristic lining of the floor and walls of the branching pyloric stomach (Fig. 14). Zymogen cells are numerous throughout, but particularly in the radial reservoirs (Fig. 15). All of these areas also contain very abundant mucous cells, varying from tall, slender goblets with fine granular secretion to large, flask-shaped glands filled with coarse, homogeneous globules, resembling those encountered in the radial gutters. These may represent different secretory phases of a single basic type of mucous gland. In the central folds between radial branches of the pyloric stomach the epithelium exhibits an interesting feature; interspersed among the upper ends of the tall, crowded epithelial cells lie small, spindle-shaped bodies staining deeply with the hematoxylins or with carmalum. They increase in abundance in the folds of the radial reservoirs and are particularly numerous and conspicuous in areas where opposite side walls are closely apposed (Figs. 16, 17a, 17b). They are, at least in these areas, almost entirely limited to the upper third or quarter of the epithelium and so are in reasonable proximity to the lumen. These elements resemble most strongly the bodies described by Smith (1937) as primary sense-cells in *Marthasterias;* they are very much like the sense cells Smith found in the radial nerve cord and bear a less striking resemblance to the cigar-shaped nuclei of the cells he interprets as representing the same elements in the lining of the stomach (see also Anderson, 1954). The deeply-staining spindles in my preparations of *Henricia* are evidently nuclei, sometimes granular but most frequently dense and homogeneous. The cytoplasm to which they belong cannot be distinguished, crowded as these bodies are in the great profusion of secretory cells (Figs. 17a, 17b); it probably consists, as Smith's Figure 5 suggests (1937, p. 123), of slender, fibrous processes running downward from the free border of the epithelium into the nerve plexus layer below. Diligent search for similar bodies in the cardiac stomach reveals a number of spindle-shaped nuclei deep in the epithelium of the flagellated tracts, usually in association with large mucous glands (Fig. 13).

In each radial reservoir, an abrupt line of transition is evident where the richly secretory side-wall epithelium gives way to the conspicuously-flagellated typical-cell epithelium (Fig. 18) that covers the roof of the pyloric stomach and continues as the lining of the intestine.

The "granulated gland cells well-stained by eosin" mentioned by Hayashi (1935, p. 10) as a component of the epithelium in the cardiac stomach, and as being present in suddenly increasing numbers in the pyloric stomach, cannot be identified, unless they correspond to what I have called zymogen cells. In my preparations the zymogen granules stain neither with eosin nor with Harris' hematoxylin; they appear as clear, refractile spherules crowded in an eosinophilic ground cytoplasm. This, it will be noted, may offer an answer to the puzzling

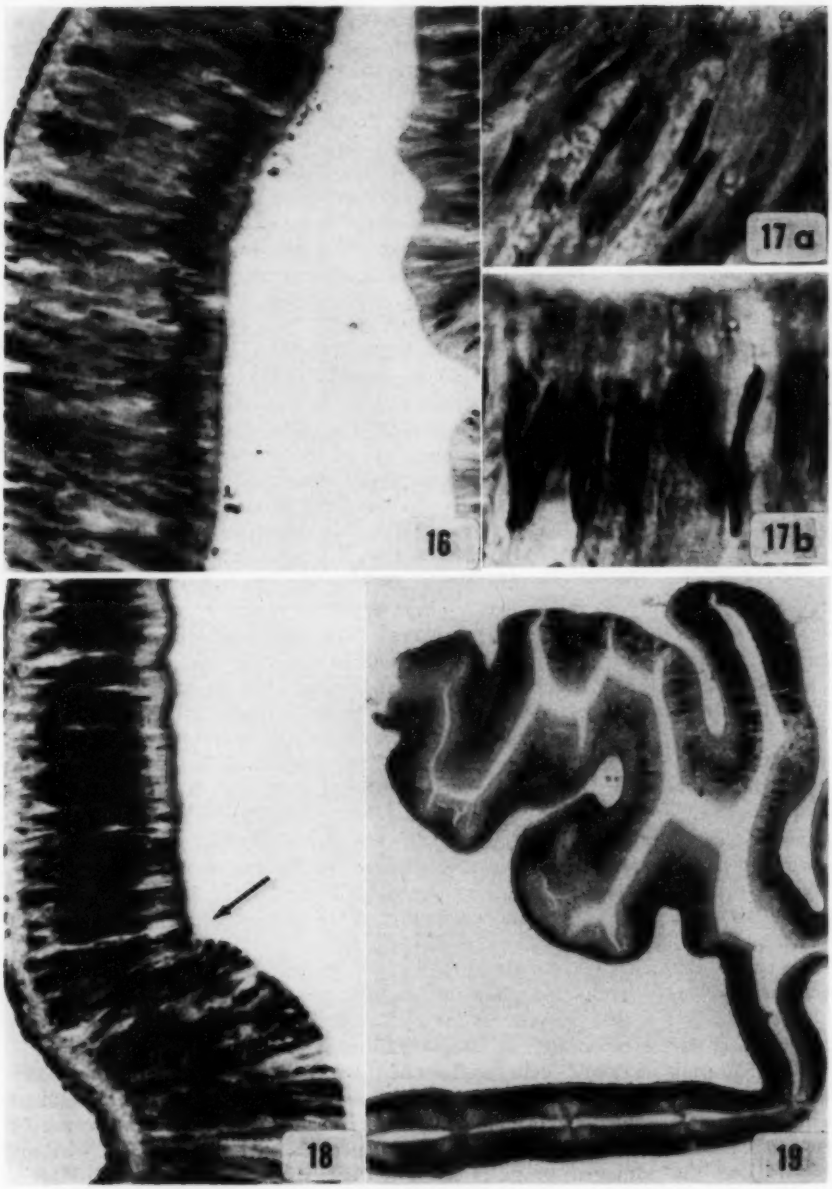


FIGURE 16. Side wall of a radial reservoir, showing abundance and localization of spindle-shaped nuclei interpreted as pertaining to sensory cells. PTAH; 470 ×.

problem: the *cells*, as Hayashi says, are granulated and do stain well with eosin; but the granules themselves are not eosinophilic.

The duality so evident in the gross anatomy of the pyloric caecum-Tiedemann pouch complex is revealed also in the histology of the organs. The caecum and its median duct share epithelial characteristics with the central pyloric stomach and its radial reservoirs, while the histological affinities of the pouch are, not surprisingly, with the lower part of the pyloric stomach and even with the radial pouches and gutters of the cardiac stomach. It will be recalled that these areas form continuous flagellated tracts in the stomach, leading directly into the openings forming the roots of the paired Tiedemann pouches. Figure 19 illustrates the striking histological contrast between the upper and lower parts of the caecum-pouch complex.

As each radial reservoir of the pyloric stomach tapers to form the median duct of its caecum, the proportion of zymogen cells in its epithelium increases to a maximum. The roof and side-walls of this median duct present a greater concentration of zymogen cells than any other part of the caecum; the folded walls of the lateral diverticula branching from the duct always contain large numbers of such cells (Figs. 19, 20), but nowhere in these lateral areas do they occur in such profusion as in the median duct. In the scattered zymogen cells of the diverticula, but not in the crowded ones of the duct, the masses or strings of granules are commonly accompanied by clear vacuoles (Fig. 21), a condition previously noted in the caecal zymogen cells of *Asterias forbesi* (Anderson, 1953) and still without explanation. The epithelium of the median duct, and to a lesser extent that of the lateral diverticula, also contains numerous mucous goblets like those found in the pyloric stomach, as well as a considerable representation of the dense spindle-shaped bodies interpreted as pertaining to sense-cells. These last are very difficult to find in the lateral diverticula and particularly so in the outer parts.

Beyond these special features, the pyloric caeca present little to distinguish them histologically from those of *Asterias forbesi* (Anderson, 1953). Most of the cells making up the epithelium of the lateral diverticula are relatively undifferentiated, with flagella and apical brush borders, and evidently function chiefly as absorptive and storage cells. Most of them contain several to many coarse, granular bodies, usually lying below the level of the nucleus (Fig. 21). These are highly basophilic and are Schiff-positive after periodic acid oxidation, resisting salivary digestion; they are not metachromatic and do not stain with Alcian Blue. Although this is insufficient for specific characterization, the staining behavior of these bodies suggests that they consist of some type of mucopolysaccharide that may be serving as a nutritional reserve. In contrast to the storage cells in the caeca of *Asterias*, those of *Henricia* appear to contain very little glycogen

FIGURES 17a, 17b. Details of sense-cell nuclei. PTAH; 1100 ×.

FIGURE 18. Sharp transition zone (arrow) between richly secretory side-wall epithelium of radial reservoir (*below*) and current-producing, flagellary epithelium of roof (*above*). PTAH; 470 ×.

FIGURE 19. Overall cross-section of a caecum-pouch complex; the pouch (*below*) normally hangs straight down (*cf.* Fig. 5) but has been bent over in histological processing. Note the marked localization of deeply-staining zymogen cells in the median caecal duct (*upper right*), and the arrangement of the parallel flagellated channels traversing the pouch from the gutter (*lower left*) to the lower part of the median duct. Seam-cell adhesions separate adjacent channels. PTAH; approximately 75 ×.

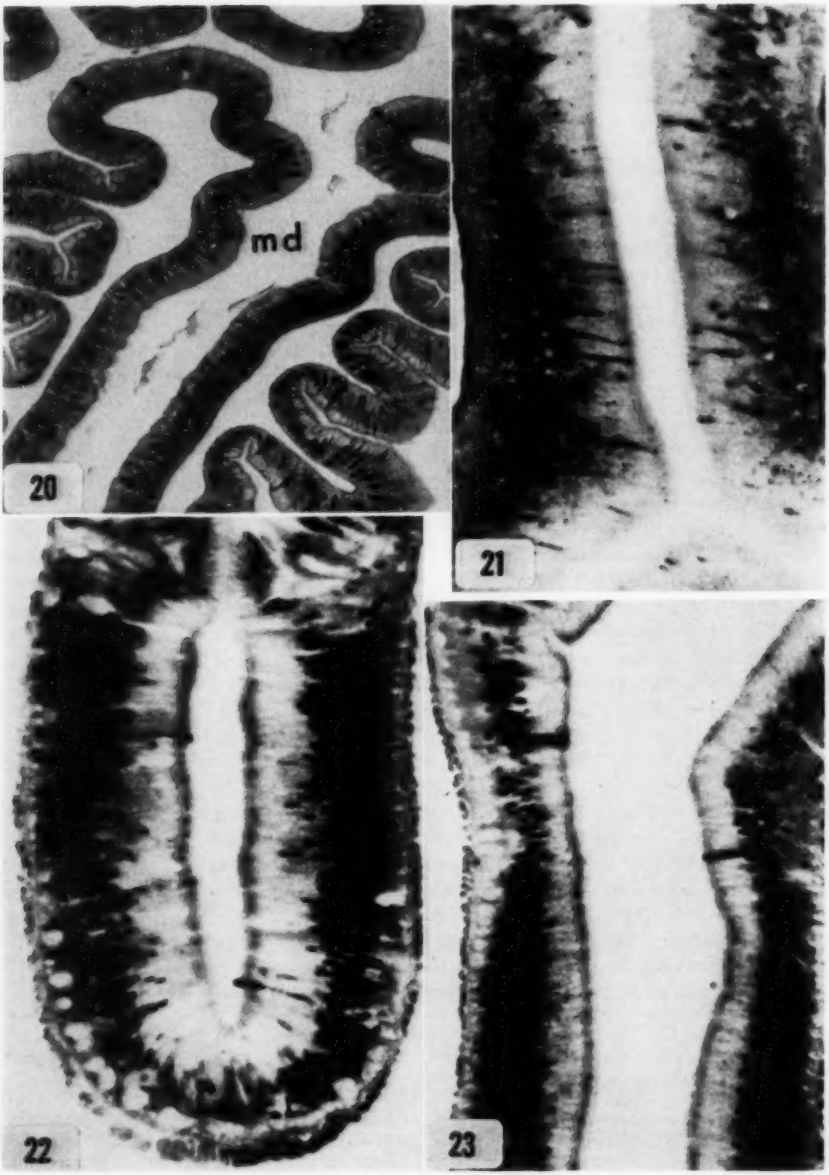


FIGURE 20. Frontal section through side wall of median duct, showing relationship between its lumen (*md*) and the lateral diverticula branching from it. Note also distribution of zymogen cells (*cf.* Fig. 19). PTAH; approximately 75 ×.

or lipid. This condition is perhaps related to the fact that my specimens of *Henricia leviuscula*, from which tissues were taken for the periodic-acid-Schiff and Sudan black technics, were collected just as they entered their breeding season. Although detailed studies involving *Henricia* have not been made, the work of Greenfield, Giese, Farmanfarmaian and Boolootian (1958) has conclusively demonstrated for two other starfishes that glycogen and particularly lipids are transferred from storage in the caeca for utilization in the ripening gonads as the breeding season approaches.

Along a line at the lower edge of the median duct, below the origins of the lateral diverticula, the epithelium changes abruptly (Figs. 19, 23). This line marks the junction between Tiedemann's pouch and the caecum proper. Within the pouch, the epithelium of the side walls is almost a pure population of "typical cells"; zymogen cells are few and widely scattered, and mucous cells, as will be seen, are strictly localized according to a definite pattern. The typical cells are extremely crowded and laterally compressed; the nuclei lie in a wide band of varying levels, a brush border is well developed, and each cell bears one or two (difficult to determine) long and powerful flagella, with conspicuous, elongate basal bodies and intracellular fibrils.

The floor of Tiedemann's pouch is narrow, formed by a gutter leading outward from one of the marginal openings low in the pyloric stomach. The epithelium lining this gutter contains an abundance of large mucous gland cells of the dense, bulbous type, their swollen cavities packed with globular secretion masses distorting their own nuclei and crowding the neighboring epithelial cells in all directions (Figs. 22, 24). Deep in the epithelium, between the mucous goblets, lie a considerable number of spindles, like those seen in similar locations in the cardiac stomach.

At regular intervals, bands of the crowded mucous-epithelial cells pass diagonally upward from the gutter to the lower margin of the caecum above, traversing the normal epithelium of the side walls of the pouch as a series of parallel striations clearly visible through the wall of the organ (see Figure 5). A transverse section of the caecum-pouch complex, such as that shown in Figure 19, cuts across several of these striations; examination reveals that each is a line of adhesion between corresponding bands of mucous-epithelial cells on facing walls of the pouch. These seams divide the pouch into approximately 30 parallel channels (in an average-sized animal), running diagonally upward from the gutter to join the median duct of the caecum, each channel lined on both its side walls by a type of epithelium evidently highly specialized for the production of powerful flagellary currents. The seams or lines of adhesion separating adjacent channels involve bands of tissue several to many cells wide, in which the nuclei in both of

FIGURE 21. Detail of epithelium in a lateral diverticulum. Note vacuoles associated with zymogen granules, and the abundant coarse granules below the nuclear level, interpreted as mucopolysaccharide deposits. PTAH; 470 ×.

FIGURE 22. Cross-section of the oral gutter of Tiedemann's pouch, bounded at top by an adhesion-seam. Note the crowded current-producing cells in the side wall, the few and scattered zymogen cells, the abundant mucous gland cells in the floor of the gutter (*cf.* Fig. 24), and the thickened tracts (clear basal circles) of the nerve plexus layer in the floor of the gutter. PTAH; 470 ×.

FIGURE 23. Transition zone marking the junction between Tiedemann's pouch and the pyloric caecum, where the current-producing typical-cell epithelium of the pouch gives way to the secretory and storage-cell epithelium of the caecum. PTAH; 470 ×.

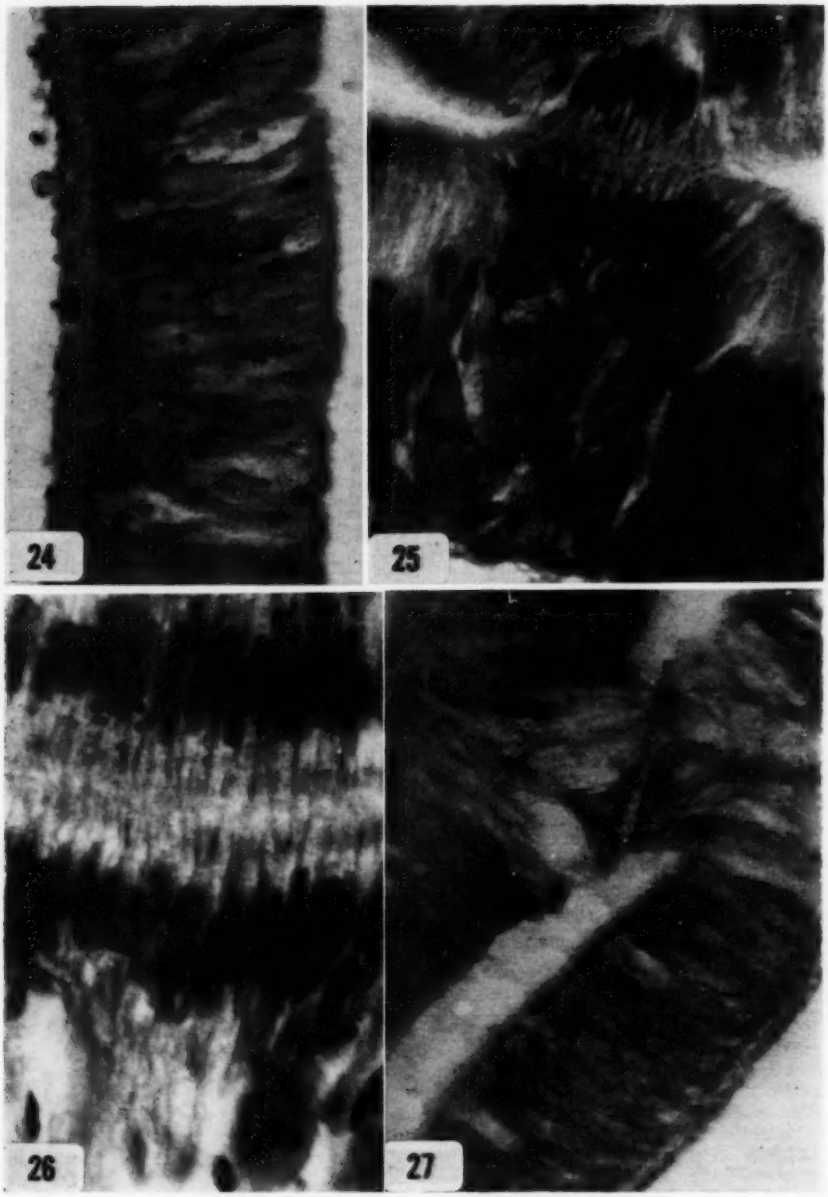


FIGURE 24. Frontal section, side wall of oral gutter in Tiedemann's pouch, to show the abundance of huge, bulbous mucous glands characteristic of this area. Note also spindle-shaped nuclei scattered deep in the epithelium. PTAH; 470 ×.

the apposed epithelia lie exceptionally high in the tissue. In section (Fig. 25) they give the impression of having been heaved or herniated outward from the basement membrane by pressure from the swelling bands of mucous gland cells between which they are confined.

The localization of the herniated seam-cells and their flanking bands of mucous cells, and the regular alternation of these zones of adhesion and the flagellated channels which they separate, are clearly shown in Figure 19. Although their nuclei have been raised almost to the level of the free edge of the epithelium, the seam-cells retain their attachment to the basal connective-tissue layer by way of elongated, compressed cytoplasmic stalks in which supporting fibrils are strongly developed. These intracellular fibrils continue upward past the nuclear level and form a characteristic and conspicuous feature of the distal ends of the joined cells (Figs. 25, 26). They do not, however, cross the plane of fusion to join the fibrils of cells in the opposite walls, and I have seen no other evidence of actual cytoplasmic continuity between the adherent cells; in favorable preparations a distinct double membrane can be observed at the seam-line. Nevertheless, the adhesion-seams are firm, close, and evidently permanent; when the tissue is broken or torn at a seam in the course of histological manipulations, it is noteworthy that the seam almost never tears at the line of fusion but rather just below the nuclei of the seam-cells of one side or the other.

The distal ends of the seam-cells contain a number of moderately coarse granular deposits which in their staining behavior resemble the Schiff-positive globules of the storage cells in the pyloric caecum. As in the caecum, very little glycogen or lipid appears in any of the cells of Tiedemann's pouch. Oddly, the only conspicuous sudanophile inclusions in the pouch areas lie in the distal ends of the seam-cells, appearing as discrete droplets scattered in the supranuclear cytoplasm (Fig. 27). The significance of lipid localization in these highly specialized and aberrant cells is problematic.

The sense-cell spindles identified deep in the epithelium of the gutter accompany the adhesion-bands also. Here they lie among the swollen mucous goblets (Fig. 25) and are often compressed against the elongate, fibrous stalks of the seam-cells. Smith has noted that in *Marthasterias* the sense-cells occur principally in those areas of the gut where the subepithelial nerve plexus layer is particularly well developed. In this connection it is of interest that in *Henricia* the floor of the gutter is underlain by several notably thickened tracts of the plexus layer (Fig. 22), and that similar thickenings accompany the specialized adhesion-tracts and unite with conspicuously well-developed areas of the plexus layer along the line of attachment between Tiedemann's pouch and the caecum (Fig. 23). The correspondence in distribution between the thickenings of the plexus layer and the

FIGURE 25. Detail of a seam-cell adhesion-band in Tiedemann's pouch. Note the apparent herniation of the seam-cell nuclei upward, and the attachment of these cells to those opposite in the epithelium across the lumen. The seam-cells maintain attachment to the basal connective-tissue layer; note the band of bulbous mucous goblets flanking the seam-cell zone on both sides, scattered sense-cell nuclei, and crowded typical-cell epithelium in channels to left and right. PTAH; 1100 ×.

FIGURE 26. Distal ends of apposed seam-cells. Note that the intracellular fibrils are not continuous across the line of fusion. PTAH; 2100 ×.

FIGURE 27. Sudanophilic droplets in distal ends of seam-cells. Sudan black-carmalum; 470 ×.

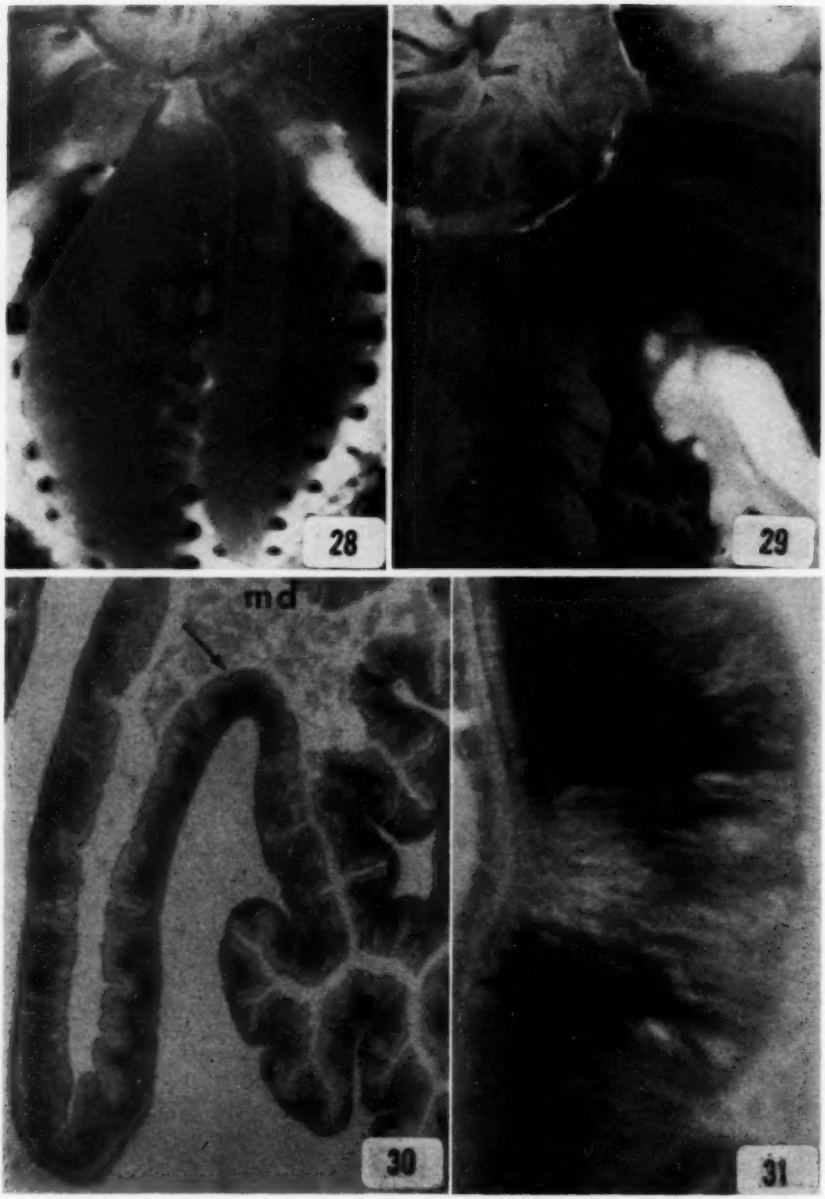


FIGURE 28. A pair of caecum-pouch units in *Patiria*, from below. Note the separate origins from the pyloric stomach, and the broad, bag-like nature of the Tiedemann's pouches. Dissection of a living specimen, approximately 5 ×.

concentrations of sense-cell spindles in Tiedemann's pouch seems unlikely to be merely coincidental.

C. *Function*

The relatively small size of the cardiac stomach, and the absence of voluminous gastric pouches, make it appear improbable that feeding in *Henricia* involves eversion of the stomach to the extent characteristic of *Asterias* or *Patiria*. Living specimens at rest are frequently observed with the mouth widely open, with folds of the lower cardiac stomach lying over the peristomial lips. Within the stomach the lumen is largely occupied by the swelling vesicles of the upper cardiac stomach, framing the narrow, stellate opening into the pyloric stomach. In some specimens, these vesicles appear partially everted through the mouth, where they form lobe-like structures extending only about a millimeter. It is to be noted that the stomach is provided with a stout muscular and fibrous retractor harness which, while differing markedly from that described for *Asterias* and *Patiria* (Anderson, 1954, 1959), still appears much too well-developed to be merely an anchor for the resting stomach. The action of the stomach suggests that the retractors function in securing the vesicular lobes, adjusting their degree of eversion, and maintaining the folds or gutters between them.

The organization of the various flagellated tracts, ducts, and channels suggests that the digestive tract in *Henricia* is particularly well adapted for the production of currents and the transport of suspended particles. The existence of such currents, and their intricate interrelationships within the digestive tract, can be demonstrated by the use of suspensions of India ink or stained yeast cells. Particles move rapidly upward through the radial pouches and gutters of the cardiac stomach, into the pyloric stomach, and out through the marginal openings into Tiedemann's ducts. In dissected specimens, suspensions introduced into one of these ducts are observed to pass distally with astonishing rapidity through the gutter of the pouch, from which successive streams are diverted and directed upward through all the diagonal flagellated channels into the median duct of the pyloric caecum above. Reaching this, particles move into the lateral diverticula of the caecum, up their side walls, and back across their roofs into the aboral part of the median duct. Here, a strong centrally-directed current streams inward, moving the suspension across the folded surfaces of the radial reservoir and into the narrow central lumen of the pyloric stomach. Currents converging here from all the radial reservoirs pass downward into the lower part of the pyloric stomach, where particles again come under the influence of the radial flagellated gutters and the currents setting towards the marginal openings and into Tiedemann's

FIGURE 29. Detail of similar structures. Note the oral gutter on the margin of Tiedemann's pouch, and the fold-patterns traversing the side walls. Approximately 10 ×.

FIGURE 30. Cross-section of a caecum-pouch complex. Note the absence of flagellated channels or adhesion seams, the relationship between the broad lumen of the pouch and the median duct (*md*) of the caecum above, and the crowded band of typical cells (*arrow*) at the junction between pouch and caecum. Zymogen cells are also visible in the epithelium of the lateral diverticula. PTAH; 75 ×.

FIGURE 31. Sudanophilia in side-wall epithelium of Tiedemann's pouch, *Patiria*. Note that the mucous-gland patch (clear center area) is flanked on each side by a band of highly sudanophilic cells which gradually fades into a typical-cell epithelium. These alternating bands are responsible for the appearance of parallel folds in the side walls. Sudan black-carmalum; 470 ×.

ducts. A complete and very active circulation through the digestive tract is thus maintained, featuring one-way transport of solutions or suspensions by specifically-directed flagellary currents. Sorting mechanisms undoubtedly occur; in the radial reservoirs, for example, selected particles are probably carried across the roof of the pyloric stomach and into the intestine for elimination. But it is clear that a given particle, until thus eliminated or until completely digested, can make innumerable circuits through the secretory regions of the digestive system. There are regional differences in transport velocity—currents in the lateral diverticula of the caeca, for instance, move much more slowly than those in Tiedemann's ducts and pouches—but there appear to be no static areas and no chambers in which large masses of ingested food can lie while undergoing dissolution.

D. Tiedemann's pouches in other starfishes

As pointed out by Cuénot (1887), members of the Family Echinasteridae (*Echinaster, Henricia*) share with those of the Family Asterinidae (*Asterina, Patiria*) conspicuous development of the organs we are calling Tiedemann's pouches. In *Patiria miniata,* for example, the paired caeca of a single ray originate separately from the pyloric stomach, and a well-developed, elongate pouch hangs from the oral midline of each caecum (Figs. 28, 29). The lower margin of the pouch is formed by a tubular gutter, taking its origin, like the similar structure in *Henricia,* from an opening in the wall of the stomach just above the circumferential fibrous girdle. As in *Henricia* also, the walls of the pouch are traversed by a regular series of parallel folds, visible externally, that run diagonally upward from the gutter to the median duct of the pyloric caecum above. The lateral diverticula of the caecum form complexly folded pockets arising at intervals from the lateral-aboral walls of the median duct. Although superficially similar, the caecum-pouch complexes in *Patiria* and *Henricia* present many fundamental differences. *Patiria* lacks, for example, the specialized portions of the pyloric stomach termed radial reservoirs in *Henricia;* Tiedemann's pouch and the median duct of the caecum open into the pyloric stomach by a common aperture, tall and narrow but not divided. Histological comparison reveals further contrasts (Fig. 30). Within the caecum, zymogen cells, mucous gland cells, and storage cells are more or less uniformly distributed, without the marked segregation of zymogen cells in the median duct so conspicuous in *Henricia.* It will be noted also that Tiedemann's pouch is more broadly attached below the caecum, and that the pouch itself is broader and more bag-like. This is explained by the fact that, as Figure 30 shows, the opposite walls of the pouch are not held together by mucous-epithelial adhesions as in *Henricia* but are only approximately parallel and not closely apposed. The conspicuous striations in the walls, superficially so similar to those of *Henricia,* are produced by parallel bands of large, crowded mucous gland cells alternating with bands of extremely crowded, very tall "typical" cells. A notable concentration of these non-secretory cells is always found in the zone of attachment where Tiedemann's pouch joins the median duct of the caecum. No zymogen cells are found in the pouch. The distribution of lipids is significantly different from that in the pouch of *Henricia.* All of the crowded typical cells show accumulations of sudanophile droplets in their basal portions, even extending through the thin strands that penetrate the thick nerve plexus layer and

insert on the connective-tissue sheet. (Fig. 31). The mucous gland cells contain no notable lipid deposits, but each of the mucous bands is flanked by strips in which the typical cells contain exceptionally high concentrations of lipid droplets. These fade gradually into the normal typical cells in the areas between the mucous bands. There are no cells here comparable to the herniated seam-cells of *Henricia* with their apical concentrations of sudanophile droplets.

Suspensions of stained yeast cells introduced at the opening leading into one of the pouches are carried through a pattern of circulation very similar to that observed in *Henricia*. Even though the walls of the pouch do not adhere to form separate channels, the concentrated flagellary bands are capable of producing rapid currents that distribute materials along the entire length of the median duct. There are no quiet or stagnant areas; whatever enters the pouch moves immediately into the caecum, then back to the pyloric stomach and outward through the pouch again. The general pattern described by Irving (1924) in the caecum-pouch complex is verified, although the anatomical relationships shown in his figure (p. 117) are inaccurate.

Without going into detail, brief anatomical and histological examination of the digestive tract of *Asterina gibbosa* shows that the features of its pyloric caecum-Tiedemann pouch complex are very similar to those of its close relative *Patiria* and differ in the same ways from those of *Henricia*.

A figure sketched by Richters (1912) in his study of regeneration in *Linckia* led me to suspect that the pyloric caeca of this starfish might also bear Tiedemann's pouches, although this has not been reported in the literature and *Linckia* is placed in a different order (Phanerozonia) from that to which the asterinids and echinasterids belong (Spinulosa) (see Hyman, 1955, pp. 334, 336–337). Preliminary studies on preserved specimens of *Linckia guildingi* reveal that Tiedemann's pouches are indeed present and are larger and better-developed than those of any other species examined so far. They extend almost to the distal ends of the long pyloric caeca, and their proximal ducts are separated by partitions from specialized portions of the pyloric stomach resembling the radial reservoirs of *Henricia*. Transverse sections of the pouches show that adhesion-bands similar to those of *Henricia* divide the pouch into separate flagellated channels. The gutter forming the oral margin of the pouch is divided by a fold arising from its floor, which externally encloses a stout band of muscle fibers. Altogether, although details have not been exhaustively studied, it is evident that the caecum-pouch complex in *Linckia* is strikingly similar in its fundamental characteristics to that described in *Henricia*. This is the more interesting in view of the wide taxonomic gap that apparently separates the two genera.

Astropecten, in which Tiedemann (1816) originally described the pouches that now bear his name, has very small ones extending a centimeter or less along the oral side of the median duct of the caecum. Their side walls show a few moderately developed parallel striations, but in the preserved material on which I have made a preliminary study these do not form firm adhesions across the lumen.

Discussion

There are many anatomical and histological differences between the organs of the digestive tract in *Henricia* and those of other starfishes in which details

have been described. The small size of the cardiac portion of the stomach, the large size and peculiarly branched structure of the pyloric portion, the conspicuous development of the rectal caeca, and other features, were noted by Hayashi (1935). Further, the cardiac stomach of *Henricia* lacks any hint of the elaborately branching patterns of flagellated gutters, with accompanying ramifications of the retractor strands and corresponding localizations of specialized epithelial cells, characteristic of the cardiac stomach in *Asterias, Pisaster, Pycnopodia,* and *Patiria* (Anderson, 1954, 1959). Lacking these, the stomach of *Henricia* is not, however, without specializations of its own, such as the so-called esophageal pouches and the alternating gutters and vesicles at higher levels, with their marked histological distinctions. Other histological peculiarities are common in various parts of the digestive tract. For instance, areas in the cardiac stomach and almost the whole of the pyloric stomach are lined with zymogen cells; in contrast, the zymogen cells of *Asterias forbesi* and *Patiria miniata* are found only in the pyloric caeca. Within the caeca of *Henricia,* the startling concentrations of zymogen cells in the walls of the median ducts are unique; in *Asterias,* this area is given over to a population of tall, crowded, current-producing cells interspersed with mucous goblets, and the zymogen cells are restricted to the lateral diverticula.

Such instances of structural contrast could be multiplied, all leading to the conclusion that in starfishes, as in other animals, corresponding parts of the digestive system, clearly homologous, may be adapted to diverse functions in relation to differences in food and in feeding methods. The food habits and feeding mechanisms of *Asterias* and similar forms are reasonably well understood; that they are probably not at all like those of *Henricia,* which are unknown, is indicated by the conspicuous structural differences revealed by detailed study.

In general, the specializations noted in *Henricia* seem to involve a tendency to segregate tissues with predominantly secretory functions in areas more or less distinct from those serving primarily for current production. Further, while the transport of materials through the gut by flagellary currents is common to all starfishes, in *Henricia* this function has been raised to a peak of efficiency by the development of the special modifications of the pyloric stomach called Tiedemann's pouches. By means of these elaborate organs, suspensions or solutions brought to them by the flagellary tracts of the stomach are very rapidly distributed to the secretory and absorptive areas of the pyloric caeca and pyloric stomach, and maintained in repeated circulation through these areas until digested, absorbed, or eliminated.

Studies elucidating the current-patterns maintained within the digestive tract have been made on a variety of asteroids, including *Asterias rubens, Solaster papposus, Porania pulvillus,* and *Astropecten irregularis* (by Gemmill, 1915); *Patiria miniata* (by Irving, 1924); and *Asterias forbesi* (by Budington, 1942). It is noteworthy that in all of these species, of diverse taxonomic affinities, the patterns are reasonably similar in general outline, with minor variations related to anatomical differences. In all, currents flow in the oral-aboral direction from cardiac to pyloric stomach; radially across the floor of the pyloric stomach and outward along the oral sides of the pyloric ducts, continuing on the oral side of the median duct in each caecum; in a circular fashion through the lateral diverticula and back to the median duct, this time aborally; and finally returning toward the

pyloric stomach again in the aboral part of this duct. In the Asteriidae, particularly, the pyloric duct is a restricted passage; within it, however, currents must be maintained both centrifugally and centripetally, the one current running on its oral side, the other on its aboral side.

It is obvious that the feeding and digestive activities of the predatory, carnivorous starfishes in this family place no demands upon their transport mechanisms that are not satisfied by the system as it exists. Yet, if one speculates upon ways in which this system might have developed to operate more efficiently for the maintenance of directed currents in the standard pattern, one is likely to be struck first by the existence of the bottle-neck at the pyloric duct. For increased efficiency, the pyloric duct should be larger, it would seem, or at least taller, so that the centrifugal and centripetal streams might be more widely separated. Alternatively, a continuous partition should be provided, set horizontally between the floor and the roof of the duct, for complete isolation of the two currents. In effect, it will be noted, the pyloric ducts of *Patiria* embody the former principle, while those of *Henricia* follow the latter. These considerations seem to justify the conclusion that in both *Patiria* and *Henricia* the functions of the digestive system have placed a higher premium upon efficiency of the flagellary transport mechanisms than is the case in *Asterias* and its close relatives.

Below and distal to the partition that divides the pyloric duct in *Henricia*, Tiedemann's pouch has evolved into a hydrodynamic organ or flagellary pump of prodigious effectiveness. Its design involves close-set, strongly-flagellated side walls, strengthened, reinforced, and held at what we may assume to be an optimal distance from one another by the unique mucous-epithelial seams, which also break up the otherwise broad epithelial expanse into channels set in regular parallel array. These features make it possible for the organ to develop what must be a considerable suction, as the force developed in all of the 30 or so flagellated channels is transmitted to the upper cardiac and lower pyloric stomach by way of the restricted Tiedemann's duct. Fluids and suspensions are drawn rapidly from the stomach through this duct and are ejected at reduced velocity along almost the entire length of the median caecal duct, into which the channels of Tiedemann's pouch lead. It is reasonable to assume that the development of the 10 Tiedemann's pouches as organs furnishing the principal motive power for circulation through the entire digestive tract brought about the emancipation of areas in the pyloric stomach, pyloric ducts, and median caecal ducts from the primarily flagellary functions they must serve in the Asteriidae, and their development of the richly secretory epithelium that is their chief characteristic in *Henricia*.

In contrast, Tiedemann's pouches in *Patiria* are, as we have seen, broad and bag-like, with no adhesion-seams to hold their side walls in apposition and only their similarities in origin and anatomical relationships to suggest that they are related to the pouches of *Henricia*. The alternating diagonal bands of mucous glands and typical flagellated cells undoubtedly approach in functional significance the parallel channels of *Henricia*, to which they show a superficial resemblance in external appearance. Irving (1924) concluded that Tiedemann's pouches in *Patiria* represent regions specialized for distribution, and all observational evidence bears out this supposition. It is to be doubted, however, that these relatively crudely-developed

organs even approach in efficiency the more elaborate ones of *Henricia*. The enlargement of the pyloric duct and the median duct of the caecum resulting from the development of the pouch in *Patiria* must represent an advance over the condition that exists in *Asterias*, but one is led to the conclusion that as organs of distribution, or flagellary pumping organs maintaining circulation within the digestive tract, the pouches of *Henricia* are far superior to those of *Patiria*.[2]

Many features of anatomy and histology in the digestive system of *Henricia*—the small size and doubtful eversibility of the cardiac stomach, the absence of any sizable chambers in which masses of food might lie while undergoing digestion, and the obviously high degree of specialization of mechanisms for maintaining flagellary circulation, among others—suggest most strongly that this animal is dependent in its nutrition upon suspended particulate matter rather than upon predation or any other form of macrophagy. The following experiment was performed as a preliminary test of this hypothesis. Two specimens of *Henricia sanguinolenta*, which had been maintained in an aquarium for 6 weeks, apparently without feeding, were placed in a fingerbowl containing a cloudy suspension of *Mytilus* sperm in sea-water, to which had been added a quantity of minute particles of Nile blue sulfate (sparingly soluble in sea-water). Externally, streams of particles could be observed converging upon the mouth, most rapidly and conspicuously in the ambulacral grooves. The mouths of both specimens remained open, much as in Figure 1, and while coarse clumps or large particles were rejected, smaller particles were either swept directly through the radial peristomial grooves at the angles of the mouth or entangled in strands or sheets of mucus which also moved into the stomach. After 12 hours' exposure to the suspension the animals were carefully washed to remove adherent particles and then dissected for examination of the digestive tract. In both specimens, concentrations of dye particles were found at various places in several of the Tiedemann's pouches.

Such a crude experiment should not be interpreted as having established the fact that in its normal nutrition *Henricia* is dependent upon a flagellary-mucous feeding mechanism or that this suffices for all its needs. The experiment does, however, provide a demonstration that at least under certain conditions particulate matter suspended in the surrounding water finds its way into the digestive tract. Further,

[2] I take this opportunity of clearing up a misunderstanding involving functional relationships between the pyloric caeca and Tiedemann's pouches in *Patiria*. Irving (1926) performed digestion experiments with isolated surviving caeca of this species and concluded that these organs are capable of digesting gelatin and absorbing amino acids. Hyman (1955, p. 385) questions this interpretation on the ground that the pyloric caecum of *Patiria* ". . . is underlain on the oral side by an extensive stomach diverticulum (so-called Tiedemann's diverticulum . . .), so that in Irving's experiments the gelatin solution went into this stomach diverticulum and the resulting digestion is probably to be attributed to the stomach." It must be pointed out that in *Patiria* the cavity of the pouch is everywhere completely open to, and in free communication with, the median duct of the caecum, so that anything going into the one is very soon distributed and thoroughly mixed in all parts of the other. The stomach of *Patiria* contains a vanishingly small proportion of zymogen cells, and it has been experimentally established that digestion does not occur in the stomach of an animal that has been deprived of all its caecum-pouch complexes (Anderson, 1959). Zymogen cells do not occur in Tiedemann's pouches in *Patiria*, and the only apparent source of digestive enzymes in this species is in the epithelium of the pyloric caeca themselves. Therefore, while Hyman's criticism was certainly justified in the light of information then available, the facts that have subsequently been established make it clear that Irving's conclusions cannot properly be questioned for the reasons advanced by Hyman.

it shows that in the intact animal particles so ingested pass into the radial parts of the system by pathways previously deduced on anatomical and histological grounds and on the basis of experiments with excised organs. In the absence of any other information on food and feeding in *Henricia*, all the indirect evidence that can be brought to bear seems consistent with the conclusion that finely-divided particulate matter in suspension, exploited by a flagellary-mucous mechanism, is significant in the nutrition of this starfish. If this is valid, then the very close and detailed structural correspondence between the digestive system of *Henricia* and that of *Linckia*, particularly in relation to the highly specialized Tiedemann's pouches, makes it reasonable to extend this conclusion to *Linckia* also. I can find no information on the food or feeding habits of *Linckia guildingi*.

Flagellary-mucous feeding of the type suggested for *Henricia* (and *Linckia*) is not unknown among starfishes. The experiments and extensive observations of Gemmill (1915) on *Porania pulvillus* demonstrate that alone among the starfishes tested, individuals of this species are capable of maintaining themselves for long periods on a diet of suspended particles only. The external and internal currents by which this diet is manipulated are apparently similar to those described in *Henricia*. Gemmill makes no mention of Tiedemann's pouches in *Porania*, and I have been unable to determine from the literature whether they are present. The powerful currents produced by this starfish suggest the presence of some type of specialization of the general nature of the Tiedemann's pouches in *Henricia*.

Several species of starfishes known to be chiefly macrophagous have been suspected of utilizing particulate matter, also, to some extent. It is interesting that these same forms possess more or less well-developed Tiedemann's pouches. For example, Gemmill (1915) finds that flagellary currents carry considerable amounts of particulate food into the digestive tract of *Astropecten*, a sand-dwelling starfish well known as a voracious carnivore. The behavior of *Patiria miniata*, particularly with regard to its habitual exposure of the huge, everted cardiac stomach in the external environment (Anderson, 1959), strongly suggests that it may in this way collect significant amounts of particulate matter to add to its extremely varied diet. *Astropecten*, it will be recalled, has relatively small Tiedemann's pouches, while those of *Patiria* are much larger but of simple construction compared with the elaborate pouches of *Henricia* and *Linckia*. The significance of these structures may lie in the enhancement of circulatory efficiency which even small or simple pouches may contribute, in relation to feeding currents. The marked structural similarity between the digestive tract of *Patiria* and that of *Asterina gibbosa* (which according to MacBride, 1906, subsists principally upon sponges and ascidians) is strongly suggestive of extensive functional correspondences, perhaps including particulate feeding.

Resemblances involving visceral organs, and particularly those of the digestive tract, are usually considered less reliable as indices of taxonomic relationship than, for example, similarities in skeletal structures or other hard parts. Nevertheless, it is interesting to consider from this standpoint the fragmentary information available concerning the distribution of Tiedemann's pouches in starfishes. The similarities between the pouches of *Patiria* and those of *Asterina* are to be expected in genera belonging to the same family. Although the flagellary pumping organs of the *Patiria* type superficially resemble those of *Henricia* and both are called Tiede-

mann's pouches, they are fundamentally very different structures. The resemblances are so superficial, and the structural differences so profound, that one is led to conclude that in the Asterinidae and Echinasteridae these organs have evolved independently and convergently as solutions to the problem of increasing circulatory efficiency within the digestive tract. These two families, it may be noted, are by all accounts closely related members of the Order Spinulosa.

In contrast, Tiedemann's pouches and other features of the digestive tract in *Linckia* are astonishingly similar, at least in the material available to me for preliminary study, to those of *Henricia*. The similarities are so striking and extend to such details as to make it appear unlikely that they could have arisen convergently in unrelated lines of starfishes. Yet the Linckiidae, chiefly on the basis of skeletal features that are fundamental in asteroid taxonomy, are placed nowhere near the Echinasteridae but among the valvate Phanerozonia. However, MacBride (1906, p. 471) makes the following statement about the Linckiidae, interesting in the present context: "It is possible that these forms, so different in many respects from the other families of the order, may have been directly derived from the long-armed Echinasteridae." My observations on structural details of the digestive system seem strongly to support the idea of a closer relationship between these families than is indicated by the criteria commonly used in starfish systematics.

It is obvious that any attempt to survey similarities and differences in organ systems, and particularly to trace the distribution of anatomically and histologically specialized visceral structures, among the various families and orders of starfishes will be meaningless until it can be based upon vastly augmented data. These data accumulate slowly, requiring careful and detailed study of the internal anatomy of many species on which the literature to date offers only descriptions of external appearance, skeletal features, geographical distribution, and the like. The present attempt at a comparative study of Tiedemann's pouches in a small group of starfishes is inadequate, based as it is upon insufficient information. At least, however, it provides a tantalizing glimpse of the surprisingly broad areas of knowledge still awaiting exploration and interpretation in the Asteroidea.

Summary

1. Detailed study of the digestive system in *Henricia* reveals that as a result of regional differentiation, particularly involving the lining epithelium, areas specialized for zymogenic and mucous secretion are segregated from other areas adapted for current-production. Secretory areas include the five interradial pouches and vesicles of the cardiac stomach, the pyloric stomach generally but particularly its radial reservoirs, and the median ducts and lateral diverticula of the pyloric caeca. Current-producing areas include the five radial pouches of the cardiac stomach and the gutters leading upward to marginal openings low in the pyloric stomach, and especially the very elaborate Tiedemann's pouches which spring in pairs from these openings and extend along the oral midlines of the pyloric caeca. Other starfishes, such as *Asterias*, which lack Tiedemann's pouches, restrict their zymogen cells to the lateral diverticula of the caeca and crowd current-producing cells into the median caecal ducts, an area which in *Henricia* contains an extremely high concentration of zymogen cells.

2. Tiedemann's pouches in *Henricia* are divided into numerous parallel flagellated channels leading diagonally upward into the pyloric caeca. These channels are separated by unique partitions formed by adhesion-seams between opposite side-walls of the pouch. It is evident from their structure and anatomical relationships, and has been experimentally demonstrated, that Tiedemann's pouches are flagellary pumping organs of great effectiveness. They produce currents capable of drawing suspensions or solutions from the stomach and delivering them rapidly along almost the entire length of the pyloric caeca. Centripetal currents stream back into the stomach, and thus a constant circulation of materials can be maintained through the radial secretory and absorptive areas, depending chiefly upon currents generated in the close-set channels of the ten Tiedemann's pouches.

3. The customary food and the feeding habits of *Henricia* are unknown, but several lines of evidence, anatomical and experimental, combine to suggest that this starfish, like at least one other (*Porania*), may subsist either wholly or in part upon suspended particles gathered by a flagellary-mucous mechanism.

4. In *Patiria miniata,* a species not distantly related to *Henricia* (same order, different family), Tiedemann's pouches are present and lie in about the same relationship to the pyloric caeca but are fundamentally dissimilar; they lack separate flagellated channels, the side walls being traversed only by parallel stripes of mucous cells alternating with bands of typical cells, and thus are more bag-like in general structure. Although they function similarly to those of *Henricia,* these much simpler pouches are probably less effective in current production. *Asterina gibbosa,* closely related to *Patiria,* has *Patiria*-type Tiedemann's pouches. *Astropecten,* in which these pouches were originally described (1816), has relatively small ones of simple construction. Both *Patiria* and *Astropecten* have been suspected of supplementing their macrophagous diet by flagellary-mucous particle-feeding, and although their pouches are far less elaborate than those of *Henricia* they are probably of significance in this connection.

5. It is disconcerting to find that *Linckia,* not at all closely related to *Henricia* (different order), nevertheless has Tiedemann's pouches and other specializations of the digestive system similar in most respects to those of *Henricia.* The differences between the pouches in *Henricia* and *Patiria* are so fundamental as to suggest that they represent independently evolved solutions to the problem of increasing circulatory efficiency within the digestive tract. In contrast, the pouches of *Henricia* and *Linckia* resemble each other so strikingly that it is difficult to conceive of them as having been produced by convergent evolution.

6. It is pointed out that detailed studies on many species of starfishes now known only from external anatomy and skeletal features of preserved specimens will provide information upon which to base broader and more meaningful comparative surveys of internal specializations.

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THE FEEDING BEHAVIOR AND RESPIRATION OF SOME MARINE PLANKTONIC CRUSTACEA [1]

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Interest in the respiratory rate and food requirements of the marine zooplankton probably dates from the studies of Pütter (1907, 1909), but data are presently available for only a dozen or so species of neritic copepods and for *Euphausia pacifica* (Lasker, 1960). *Paraeuchaeta norvegica*, studied by Raymont and Gauld (1951), is the only marine copepod for which respiratory measurements are available, which commonly occurs at depths greater than 100 meters.

In the present work a number of shipboard and laboratory experiments were performed with oceanic species of copepods, amphipods and euphausids, some from deep water, to learn if such organisms were amenable to artificially controlled conditions, and to obtain additional respiratory data for a very important but little-studied group of animals. A few species previously investigated from other localities (*i.e.*, *Calanus finmarchicus*) were also included for comparative purposes.

In addition to the respiratory measurements, representatives of most species investigated were also kept in laboratory culture vessels where observations were made on their behavior and food habits. Some individuals of the copepod, *Calanus hyperboreus*, have been maintained in the laboratory for approximately one year which would seem to be the life span for it. Because of its large size, long life span, and ability to adapt to laboratory conditions, this species is currently the subject of intensive experimental investigation.

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Materials and Methods

The zooplankton organisms used in the present program included the copepods: *Calanus finmarchicus, C. hyperboreus, Paraeuchaeta norvegica, Pleuromamma robusta, Bathycalanus* sp., *Rhincalanus nasutus, Euchirella rostrata;* the amphipods *Phronima* sp., *Euthemisto compressa,* and *Hyperia galba,* and an unidentified euphausid probably belonging to the genus *Thysanoessa.* All these animals are of

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large size and easily recognizable with the naked eye, even on shipboard. For the graphic analyses of respiratory rates use was also made of some data, hitherto unpublished in the present form, for the small neritic copepod *Acartia clausi*.

The animals were captured with a ¾-meter or 1-meter net of mesh size #00 or #000, with a glass jar of one quart capacity secured in the cod-end. Tows were generally 15 to 30 minutes at depth, but in the case of the deeper tows the time required to reach the desired depth and to recover the net again increased the total towing time appreciably. Before the net was brought aboard after fishing, care was

TABLE I

Summary of all experimental data on the respiratory rates of zooplankton

Copepods	Location of experimental tow and estimated depth	Date	No. & stage	Experimental temp. ° C.	Dry weight/animal mg.	Respiration rate:	
						µl./animal & day	µl./mg. dry wt. & day
Calanus finmarchicus	Georges Bank 41°00′ N 67°35′ W 25 m.	Aug. 18, 1958	4 V	7.5	0.188	4.4	23.0
		Aug. 18, 1958	4 V	7.5	0.188	5.4	28.8
		Aug. 18, 1958	15 V	7.5	0.232	5.5	23.3
		Aug. 18, 1958	4 ♀	7.5	0.211	9.5	44.1
		Aug. 18, 1958	15 ♀	7.5	0.214	9.0	42.0
		Aug. 18, 1958	4 V	18.5	0.188	9.0	47.3
		Aug. 18, 1958	4 V	18.5	0.188	8.9	51.9
		Aug. 18, 1958	15 V	18.5	0.199	8.7	42.0
		Aug. 18, 1958	4 ♀*	18.5	0.211	15.7	74.2
Calanus finmarchicus	Gulf of Maine 42°25′ N 69°47′ W 200 m.	Aug. 20, 1958	15 V	8	0.294	4.7	16.2
		Aug. 20, 1958	15 V	8	0.264	3.9	14.8
		Aug. 20, 1958	15 V	8	0.334	3.6	10.9
		Aug. 20, 1958	4 V	8	0.277	5.4	19.5
		Aug. 20, 1958	4 V	8	0.277	4.7	16.9
Calanus hyperboreus	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	7 V	<10	1.55	18.3	11.8
			5 ♀	<10	3.68	24.8	5.5
			1 ♀	<10	3.62	26.0	7.1
Paraeuchaeta norvegica	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	5 ♀	<10	3.88	47.6	12.2
			5 ♀	<10	3.80	51.5	13.8
			1 ♀	<10	4.59	48.7	10.5
Pleuromamma robusta	Slope water 38°28′ N 70°59′ W 450 m.	Aug. 15, 1958	3 ♀	8	0.283	19.0	66.5
			3 ♀	8	0.283	15.5	54.3
Bathycalanus sp.	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	1 ♀	5.1	17.9	163.4	9.1

* 3 animals died.

TABLE I—*Continued*

Copepods	Location of experimental tow and estimated depth	Date	No. & stage	Experimental temp. °C.	Dry weight/animal mg.	Respiration rate:	
						µl./animal & day	µl./mg. dry wt. & day
Rhincalanus nasutus	Slope water 39°48′ N 71°12′ W 400 m.	April 22, 1959	2 ♀ 2 ♀ 2 ♀	6.5 6.5 6.5	0.826 1.079 1.019	13.0 15.3 15.0	15.7 14.2 14.7
Euchirella rostrata	Slope water 39°48′ N 71°12′ W 400 m.	April 22, 1959	3 ♀ 3 ♀ 3 ♀	6.5 6.5 6.5	0.890 0.947 0.820	22.4 28.6 25.6	25.2 34.9 27.0
Acartia clausi	Long Island Sound 5–10 m.	July 22, 1953 Jan. 6, 1954 Jan. 26, 1954 Feb. 2, 1954 July 3, 1954 July 10, 1954	119 ♀ 47 ♀ 156 ♀ 49 ♀ 100 ♀ 52 ♀	5.0 5.0 5.6 5.0 5.9 5.9	0.0041 0.0047 0.0073 0.0072 0.0044 0.0049	0.30 0.37 0.67 0.69 0.40 0.22	73.1 78.7 91.8 95.8 90.9 42.6
Euphausids (unidentified)	Slope water 41°46′ N 65°28′ W 1000 m.	Aug. 19, 1958	3 ** 1	5.1 5.1	2.67 3.39	43.1 105.7	16.1 31.0
Amphipods *Phronima* sp.	Slope water 38°28′ N 70°59′ W 450 m.	Aug. 15, 1958	1	8	27.3	284.9	10.4
Euthemisto compressa	Gulf of Maine 42°25′ N 69°47′ W 200 m.	Aug. 20, 1958	4 4 4	4 4 4	4.97 4.87 4.35	92.7 87.5 72.3	18.6 18.0 16.6
Hyperia galba	Gulf of Maine 42°35′ N 69°35′ W 250 m.	Dec. 4, 1959	1 1 1 1	5.6 5.6 5.6 5.6	3.52 8.24 4.62 10.15	51.2 81.0 70.7 114.2	14.5 9.8 15.3 11.2

** 1 animal in poor condition.

taken to have on hand fresh sea water pre-cooled to a temperature at or near that of the depth fished. The cod-end was immersed in this water and the contents of the glass jar only were retained. It was assumed that animals crushed against the meshes of the net were likely to be damaged, so that the net was never washed down and the entire process of getting the organisms from their natural temperature conditions to conditions simulating them was carried out as rapidly as possible. If the temperature difference between the surface water and the depth fished was great,

the mortality among the deep-water forms was high despite the precautions taken. However, the animals were sorted immediately and only the healthy specimens kept for experimental work.

On shipboard, a portable refrigerator which opened from the top was used as an experimental laboratory. In the earlier studies, some difficulty was encountered in regulating the temperature of this box when it was opened frequently. A larger expansion valve, enabling higher refrigerant pressures, eliminated this difficulty, and in its present form the box can be kept open for long periods even in summer without excessive temperature change. On return to land facilities, a constant temperature room and conventional refrigerated water baths provided supplementary, controlled-temperature conditions when required.

Since most of the animals used in this study came from water deeper than 200 meters, 5–6° C. was chosen as the temperature at which the experimental and observational studies would be run, although for reasons mentioned above this was not attained in every case (see Table I). Initially it was hoped to compare the respiration of some of the animals at 5° and at surface temperature, to enable the computation of a rough Q_{10}. However, prolonged exposure to warmer surface waters proved lethal to most of the experimental material so that except for the relatively eurythermal *Calanus finmarchicus*, such a comparative study was not possible.

Respiration was measured using glass-stoppered bottles of appropriate size. Animals were placed in a small quantity of water in a bottle, and then the bottle and contents were flushed several times with water of known oxygen content, using a siphon arrangement with a bolting cloth screen to prevent loss of the experimental material. Control bottles were prepared in precisely the same manner except that the animals were omitted. All bottles were filled completely, taking care to insure that no air bubbles were included. As Marshall *et al.* (1935) demonstrated an increase in respiration for *Calanus finmarchicus* on exposure to light, all bottles were placed in black cloth bags during the run, regardless of the experimental conditions. At the end of a suitable period of time (8 to 48 hours), single samples from the experimental and the control bottles were siphoned into smaller glass-stoppered bottles and the oxygen content determined by the Winkler method. The oxygen utilization was determined from the difference between the bottles containing animals and those without. (For a detailed description of the method, with a discussion of its advantages and disadvantages, see Conover, 1956, 1959.)

The animals themselves were generally dried for weighing while in fresh condition, or in some instances a sample of the same species and stage of animal was dried as representative of the experimental animals. Animals were weighed on a suitable quartz helix microbalance made by the Microchemical Specialties Company, Berkeley, California. In the case of the smaller organisms such as *Calanus finmarchicus* a balance with working sensitivity of 2 mg. $\pm$ 1 μg. was used. For larger forms a 20 mg. $\pm$ 10 μg. balance was employed. Aside from the accuracy of weighing in this manner, the process is extremely rapid and largely free from errors due to sudden temperature change, varying humidity, etc. In a few instances, the animals were too large for either helix and they were weighed on a conventional analytical balance.

In the respiration experiments, as well as in laboratory culture studies, antibiotics

were used to control bacterial growth and respiration. Dihydrostreptomycin sulfate in concentration 50 mg./L. was generally used in respiratory studies, sometimes supplemented with 10 mg./L. of chloromycetin. Chloromycetin was found to have an inhibitory effect on the feeding of *Calanus finmarchicus* when used at 50 mg./L. (Conover, Marshall and Orr, 1959). Since oxygen utilization attributable to bacteria was generally less than 0.1 ml./L., even in 48-hour experiments, with streptomycin alone, chloromycetin was eventually eliminated altogether from the experimental procedure.

For laboratory culture experiments, the organisms were kept in polyethylene "freezer containers" of pint, pint and a half, quart, or gallon size, depending on the size and number of animals to be cultured. These containers proved non-toxic to all animals tested, and, when fitted with their plastic tops, were safe from spillage or breakage in a rough sea. For most organisms, one or two animals in the pint-sized container proved most satisfactory; the containers then could be conveniently stacked four or five high without danger of upsetting, and in this size the sides are low enough to permit easy observation with a dissecting microscope.

Sea water for cultures was passed through a type AA Millipore filter (pore size 0.80 μ), cooled, and aerated before use. In the earlier studies, the culture water was generally taken from the same area that produced the animals, but it was found that local water from Vineyard Sound was also satisfactory. Streptomycin and penicillin "G" potassium 50 mg./L. were used together at first, but later alternated at each change of culture medium to lessen the possibility of "antibiotic resistance" developing among the contaminating bacteria.

The phytoplankton organisms tried as food for the animals included *Skeletonema costatum, Thalassiosira decipiens, T. fluviatilis, Chaetoceros affinis, Rhizosolenia setigera,* and *Coscinodiscus asteromphalus,* all from laboratory cultures. Living *Artemia* nauplii and *Pinnotheres* zoeae, fresh-caught harbor copepods including *Acartia clausi, A. tonsa, Temora longicornis, Centropages hamatus, Eurytemora hirundoides,* and *Labidocera aestiva,* as well as various invertebrate larvae in the plankton were given as animal food. Bits of mussel, clam, and living and dead offshore zooplankton were also given to some of the larger carnivorous forms. The number of fecal pellets produced was used as a criterion for the amount of feeding although in a few instances change in the number of food organisms was also determined.

No attempt was made to determine the food in nature by examination of gut contents. Food passes through the animal's gut very rapidly and particularly in the case of offshore forms the gut is usually empty.

Observations on Feeding and Behavior of Animals in the Laboratory

Copepods

Calanus finmarchicus. Marshall and Orr (1955a, 1955b) have summarized what is known concerning the feeding and behavior of this species, and little can be added by this investigation. *Calanus finmarchicus* is known to eat a wide variety of diatoms, dinoflagellates, and other flagellated forms. Nannoplankton is eaten but the animal showed a decided preference for larger food (Marshall and Orr, 1955b). In addition, radiolarians, tintinnids, and crustacean remains have been

found in the gut (Marshall and Orr, 1955a). In the present study, *C. finmarchicus* ate *Skeletonema, Rhizosolenia,* and both species of *Thalassiosira.* Although it may take in animal food inadvertently, it would seem that this species is primarily a herbivore.

Calanus hyperboreus. This species is generally regarded as an arctic form, where it commonly occurs in the surface waters. It is moderately abundant in the Gulf of Maine and has been observed in the slope water at depths greater than 400 meters as far south as about 38° N. Juveniles were captured a mile south of Gay Head on Martha's Vineyard, Massachusetts, in March and April, 1958.

When first returned to the laboratory, *C. hyperboreus* generally did not eat any plant or animal food readily, but after one to two weeks healthy specimens ate all phytoplankton species presented to them, including *Skeletonema costatum, Thalassiosira decipiens, T. fluviatilis, Chaetoceros affinis* and *Coscinodiscus asteromphalus.* Many large fecal pellets were produced, often two or three millimeters long, which on microscopic examination contained some green material and abundant smashed tests of the species of diatom fed.

This species is anatomically very similar to *C. finmarchicus* and is almost certainly herbivorous in northern seas. However, it is difficult to understand how it can obtain plant food in sufficient abundance to sustain it in the slope waters south of Woods Hole, Massachusetts, in summer when the waters of the euphotic zone are too warm for it. Possibly the animal goes into a state of "quiescence" when food is scarce, which may explain why no food is taken when it is first brought into the laboratory. Recent unpublished experiments suggest that the increase in activity after some days in the laboratory is also accompanied by an increase in respiratory rate.

Sømme (1934) observed that breeding animals frequently ate their own eggs. This observation has been confirmed in the present study, but it was found that egg-eating was much greater when animals had no other food available than when abundant phytoplankton was present.

Paraeuchaeta norvegica. Lowndes (1935) examined living and preserved specimens of this large copepod and concluded that it was entirely carnivorous. The animal refused all plant food presented to it in the current work. On the other hand, some of the laboratory specimens fed on small neritic copepods readily as long as they were alive. *Acartia tonsa* and *Centropages hamatus* were taken frequently, although it is questionable whether *Paraeuchaeta* would encounter either in nature commonly. It did not eat *Artemia,* though some decapod larvae were consumed. During feeding, fecal pellets were produced which contained obvious animal fragments. If both plant and animal material were fed simultaneously, the fecal pellets produced contained only animal remains.

The maxillipeds in this animal are large, prehensile and carried far forward in "praying mantis" fashion. The actual capture of the prey was not observed but the animal would seize the end of a needle or micropipette when irritated in a manner which must closely duplicate the process of food getting. The strength of the animal was surprising and a smaller copepod would have little chance of escape once it was grasped by the maxillipeds. Curiously, some of the laboratory specimens hooked their maxillipeds over their first antennae. In this position they seemed quite helpless and unable to get free. When the maxillipeds were released

by the investigator, the animal seemed quite healthy, but usually caught the maxillipeds again in a few hours.

Bathycalanus sp. There can be little doubt that these large red copepods from deep water must be carnivorous, although no food, plant or animal, was taken during the three weeks they were kept in the laboratory. The female whose respiration was measured was over 13 mm. long with antennae reaching over 2.5 cm. from tip to tip. In swimming, the movements were generally unhurried, almost deliberate, but when disturbed the animal dashed about the culture vessel in a frantic effort to escape, frequently sustained for some seconds.

Rhincalanus nasutus. This species survived well under laboratory conditions and ate any species of phytoplankton offered (*Skeletonema costatum, Thalassiosira decipiens, Rhizosolenia setigera*). One female lived for three months before being accidentally killed, during which time she matured and laid numerous fertile eggs. Attempts to raise the nauplii were unsuccessful.

Euchirella rostrata. These robust-appearing copepods did not survive particularly well in the laboratory although a few fecal pellets were produced when the animals were fed on *Skeletonema, Thalassiosira decipiens* and *Rhizosolenia.* All were dead within five weeks of capture, but during this period several females matured and laid. The eggs, which were a deep purple, were remarkable for their size relative to the animal which produced them. The lengths of the cephalothorax for the captive animals ranged from about 2.9 to 3.1 mm., while the eggs produced measured over 0.4 mm. in diameter. In contrast, *Rhincalanus nasutus,* size range 4.2–4.7 mm., laid eggs about 230 μ in diameter, and in the still larger *Calanus hyperboreus,* cephalothorax 5.5–6.0 mm., the eggs ranged from 190–210 μ. *Euchirella* laid only a few eggs at a time and they were very buoyant. Unfortunately none of the eggs developed.

The density of this animal in the adult stage was remarkably high in contrast to its eggs. The animals did not swim continuously in the laboratory containers and when swimming ceased, they sank rapidly to the bottom. The carapace seemed unusually sclerosed for so small an animal, being hard and smooth to the point of a dissecting needle. Their movements were exceedingly rapid and they leaped about vigorously when out of water. One individual traveled a measured distance of 20 cm. in a single leap from the shallow dish, containing water several millimeters deep, in which it was being examined. Although it was not demonstrated that *Euchirella rostrata* does prefer animal food, the generally poor feeding on phytoplankton, robust anatomy and prehensile head appendages make it virtually certain that the animal is largely carnivorous.

Other copepods. Only a few specimens of *Pleuromamma robusta* were taken in near-surface tows over the continental slope and no observations on the organisms in captivity were made. The structure of the mouth parts and general morphology would suggest that it is largely herbivorous (George Grice, personal communication).

No attempts were made to culture *Acartia clausi* at this time but earlier studies (Conover, 1956) leave little doubt that it is primarily a herbivore. However, two observations of considerable interest were made on the closely related species *A. tonsa.*

On one occasion, some *A. tonsa* taken from Woods Hole harbor were given as

food to a larger carnivorous copepod (*Paraeuchaeta norvegica*) in the company of some *Artemia* nauplii. During the experiment, an *Acartia* male was found which had firmly grasped with its head appendages an *Artemia* nauplius. When both animals were transferred to another dish for observation the male released the nauplius which was seen to have its abdomen almost totally eaten. The *Acartia* was given additional *Artemia* nauplii but died shortly thereafter without any further predation.

On another occasion, *A. tonsa* was observed in the act of feeding on a culture of large *Thalassiosira decipiens* (cells 70–85 μ in diameter). A female (cephalothorax length 0.87 mm.), lying ventral side up on the bottom of the dish, was seen to grasp single *Thalassiosira* cells with rapid movements of the maxillipeds, bringing them to the mouth region. Several times the individual cell could be seen poised on the edge of the labrum for an instant before it passed inside the animal without being broken or apparently damaged in any way. Once inside, the cells could be seen through the transparent carapace like beads on a string lined up along the foregut. The cells were carried posteriorly by a series of peristaltic movements during which they continued to be discrete, undamaged cells until quite suddenly they lost their distinct outline and seemed to fuse into a mass which soon became noticeably darker in color. The material was passed posteriorly and eventually extruded as a fecal pellet. The entire process took about 30–45 minutes, depending on which cell was timed. On examination the pellet was seen to contain only shattered frustules of *T. decipiens* and some unidentified organic matter.

Amphipods

Only two *Phronima* were taken during the program and one was dried after the respiration experiment. Despite their transparent, somewhat delicate appearance, the organisms were quite dense with a tough, sclerosed exoskeleton. The specimens studied here were found free-living in the plankton but the animal is frequently found "living" in an empty test of a salp. Most probably the organism eats the salp in whose test it is found, for it would seem poorly equipped for filter feeding.

Euthemisto compressa was found to produce an occasional fecal pellet when given phytoplankton (*Skeletonema*) but was obviously much more successful with animal food. For instance, between October 3 and October 6, 1958, a single female was observed to eat four harbor copepods and two zoeae, one nearly as large as itself. Gravid females were taken in the plankton on several occasions, and even the newly hatched young seemed carnivorous, swarming all over a piece of dead euphausid given them. *Hyperia galba* likewise is largely carnivorous and was observed to eat bits of mussel (*Mytilus edulis*), smashed snail (*Littorina littorea*) as well as living and dead copepods. This species is frequently associated with *Aurelia aurita* or other large medusae and may share the food captured by its larger host, but it can be a free-living member of the plankton community as well (Bigelow, 1925).

Both *Euthemisto* and *Hyperia* are quite dense, heavily sclerosed, and strong swimmers. Curiously, their carapaces are strongly hydrophobic and despite their density and obvious strength, they are very prone to become caught in the surface film.

Euphausids

Of all the oceanic organisms tried, the euphausids seemed to be the most difficult to keep in the laboratory. Specimens taken on August 19, 1958, were all dead by September 4, before anything could be ascertained about their food habits. On other occasions, *Thysanoessa*, *Meganyctiphanes*, and *Nematoscelis* have been observed to eat phytoplankton (*Skeletonema costatum*, *Thalassiosira fluviatilis*), but the rate of consumption would seem to be much too low to meet food requirements. These animals did not survive appreciably better than the first group.

Several workers have noted that euphausids consume a variety of foods. *Nyctiphanes couchii*, a neritic species, eats diatoms and organic detritus predominantly but also catches *Sagitta*, smaller crustaceans, and is cannibalistic in the laboratory (Lebour, 1925). Similarly, *Meganyctiphanes norvegica* consumed plant detritus when it was present in the water, but also fed on *Calanus finmarchicus*, *Paraeuchaeta norvegica* and smaller copepods (MacDonald, 1927). Very probably most euphausids are omnivorous, but it is difficult to explain their extreme sensitivity to deficiencies of the laboratory environment.

Respiration in Relation to Size of Planktonic Organism

It is generally believed that the respiratory rate of poikilothermal animals is related to some power of body weight by the expression

$$R = kW^x \tag{1}$$

where R is the volume of oxygen consumed, W the body weight, k a constant for a given set of conditions, and x is the exponent, generally between 0.66 and 1.00. When oxygen consumption is plotted against weight on log log paper the data should give a straight line with a regression coefficient equivalent to x in equation (1).

Raymont and Gauld (1951) obtained a regression coefficient of 2.19 (or 2.30 with a single aberrant value removed) when log respiration was plotted against log length of the cephalothorax for four species of marine copepods ranging in length over a size range of nearly an order of magnitude. If it is assumed that weight varies as the cube of the length, then the coefficient obtained by Raymont and Gauld becomes 0.73 (or 0.77) in the form of equation (1).

In the case of nine species of small neritic copepods, Conover (1959) computed an overall regression coefficient of 0.86 for log respiration against log dry weight. For these copepods with a total size range considerably less than an order of magnitude, weight was found to vary as the power 3.17 of cephalothorax length.

The range of variation in weight of the organisms included in the current investigation is nearly four orders of magnitude and the variation in respiratory rate per organism is likewise considerable (Table I). A log log plot of respiration against weight would be expected to give a straight line relationship with a positive regression coefficient. However, if the respiration rate is first divided by the weight of the animal, the log log plot of this value R' against weight W should yield a negative regression coefficient. Of the two methods, the second shows more clearly the decrease in metabolic rate with increasing size, and its use should save a step in the calculation of energy flow and production rates.

The least squares regression on double logarithmic coordinates of the respiratory rates as μl. oxygen/mg. dry weight and day plotted against dry weight in μg., excluding only the values for *Calanus finmarchicus* at 18.5° C. and those for *Pleuromamma robusta*, gives the equation

$$\log R' = -0.35 \log W + 2.2888. \tag{2}$$

In exponential form equation (2) becomes

$$R' = 194\ W^{-0.35}. \tag{3}$$

For comparative purposes, equation (3) may be converted to the form of equation (1) by replacing R' with its equivalent R/W,

$$R = 194\ W^{0.65}. \tag{4}$$

It can be readily seen that the exponential constant in (4) is decidedly lower than that observed by Conover (1959), and also is lower than the probable exponent computed from Raymont and Gauld (1951). Weymouth *et al.* (1944) obtained a coefficient of 0.798 for *Pugettia producta* and Vinberg (1950) recorded 0.81 for

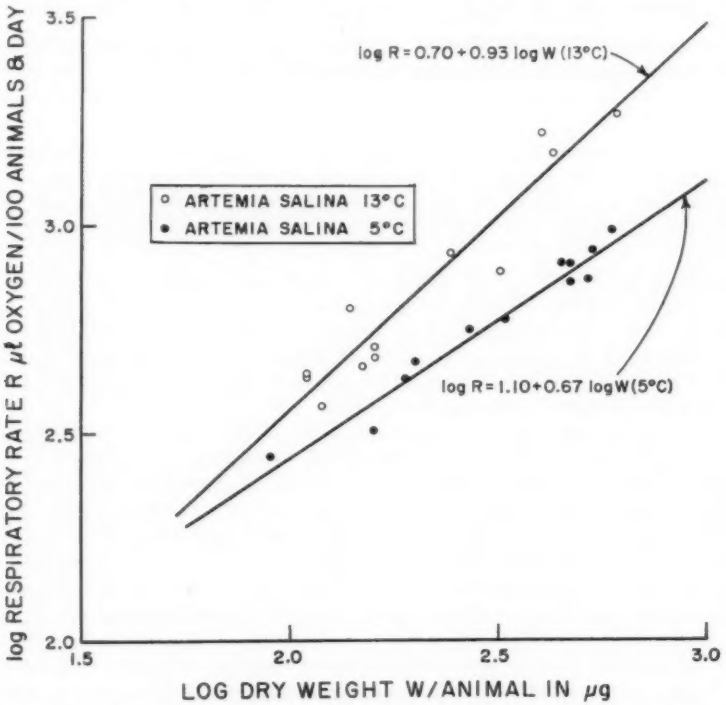

FIGURE 1. Regression lines for *Artemia salina*, showing log respiration in μl. O_2 animal and day plotted against log dry weight at 5° and 13° C.

TABLE II

Analysis of variance for regression of log respiration against log dry weight at two temperatures for Artemia salina

Null hypothesis: That there is no difference in regression coefficients for log respiration against log weight measured at 5° and 13° C.

Sources of variation		Degrees of freedom	Sums of squares	Mean square	Variance ratio
5° C.	Linear relation	1	0.319323	0.319323	173.73
	Error about line	10	0.018383	0.001838	—
13° C.	Linear relation	1	0.625688	0.625688	103.16
	Error about line	10	0.060651	0.006065	—
	Combined slope	1	0.921432	0.921432	233.17
	Between regression coefficients	1	0.023579	0.023579	5.97
	Combined error	20	0.079034	0.003952	

Variance ratio F for difference between regression coefficients = 5.97; $F_{0.975}$ (1, 20) = 5.87. Since F (1, 20) >5.87, reject null hypothesis.

Gammarus lacustris. Assuming that surface area is proportional to the square of length and that weight is proportional to the cube of length, von Bertalanffy (1951) suggested that an exponential constant of 0.667 indicates direct proportionality of metabolism to the relative surface area of the organisms. The exponential constant from equation (4) might, therefore, suggest that the surface rule applied in the case of the zooplankton investigated in the current study but not for the earlier work. However, there is also the possibility that the lower coefficient of proportionality observed here may result from the different temperature at which the experiments were run. In contrast with the temperatures of 4–8° C. used in the present work, Raymont and Gauld (1951) performed their experiments at about 17° C. and Conover (1959) used 20° as the experimental temperature. Vinberg (1950) and Weymouth *et al.* (1944) also used temperatures appreciably higher than those in the present work.

To test the hypothesis that the temperature at which a series of experiments is performed with different sized organisms might affect the proportionality of respiratory rate to size, an initial experiment with three species of calanoid copepods, ranging in weight from 0.017 to 5.45 mg., was set up at two temperatures, 5° and 13° C.; however, the scatter around the least squares regression lines was too great to permit disproof of the null hypothesis that there was no difference in the log log regression coefficient of respiration against weight at the chosen temperatures. A second experiment at the same two temperatures was then performed with a single species, *Artemia salina*, which can be cultured in the laboratory so as to supply a number of different size classes. The least square regression lines for log respiration against log weight for *Artemia* at 5 and 13° C. are shown in Figure 1. The regression coefficients, 0.67 at 5° C. and 0.93 at 13° C., can be demonstrated to be statistically different at $P = 0.025$ (Table II). According to von Bertalanffy (1951) the two regression coefficients obtained here for the same animal would be indicative of two very different metabolic types.

So long as the Q_{10} for any temperature change is the same for an animal over its entire size range, the regression of respiration against weight should give the same coefficient of proportionality regardless of the experimental temperature. In this regard, Rao and Bullock (1954) reviewed data from several sources, and concluded that the Q_{10} of various measures of activity commonly increases with increasing size over the range of ordinary physiological temperatures, although there were several cases in which the trend was reversed. *Artemia* was not one of the animals considered by Rao and Bullock, but the present data would seem to suggest that this animal does have a Q_{10} which varies with size.

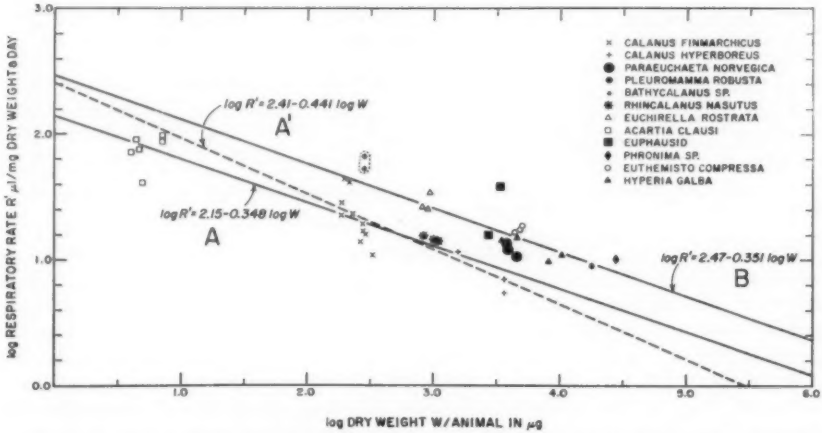

FIGURE 2. Scatter diagram and regression lines showing the relation between log respiration in µl. O_2/mg. dry weight and day and log dry weight in µg. Line A is fitted to data for suspected herbivores. Line A' is fitted to data for herbivores, omitting values for *Acartia clausi*. Line B is fitted to data for suspected carnivores. See text for further explanation.

In the case of studies involving several different organisms taken from nature, there is the additional complexity of species differences in Q_{10}. Rao and Bullock (1954) also showed that the habitat temperatures of the animal prior to examination could affect Q_{10}. In this regard, Berg and Ockelmann (1959) observed a seasonal shift in the size-respiration relationship for the fresh-water snail *Lymnaea palustris*. Other factors, such as nutritional status, reproductive activity or some endogenous rhythm, might lead to increased variability in the observations. The resultant of one or several factors of the sort described here might be to increase or decrease the spread of values at one end of the temperature scale while having the opposite effect at the other end, regardless of the size of the animals being studied. Both the slope of the regression line and the scatter of points around it would be affected.

RESPIRATION IN RELATION TO FOOD HABITS

It was noted early in the study that *Calanus hyperboreus* and *Paraeuchaeta norvegica* taken in the same tow and studied under the same conditions had different

respiratory rates despite their similarity in size and weight (see Table I). Both animals were kept in the laboratory for a lengthy period, and it became obvious that one, *Calanus hyperboreus,* was principally herbivorous while the other was entirely carnivorous. Raymont (1959) found that *Tortanus discaudatus,* also believed to be a carnivore, appeared to have a higher metabolic rate in relation to its body size than other copepods inhabiting the same type of environment.

In Figure 2 the respiratory rate at low temperatures for all the animals studied has been plotted against their weight on double log paper. On examination of the scatter diagram it seemed that the suspected carnivores in general had higher rates than the known herbivores. To test this hypothesis, separate regression lines were

TABLE III

Analysis of covariance for respiratory rates of herbivores (Acartia clausi *included*) *and non-herbivores*

Null hypothesis: That there is no difference in the respiration rate per mg. of dry body weight for herbivorous and non-herbivorous zooplankton

Sources of variation	Degrees of freedom	Sums of squares for respiration	Sums of products for respiration and weight	Sums of squares for weights	Errors of Estimate		
					Sums of squares	Degrees of freedom	Mean square
Total	38	3.76580	−10.74861	43.19223	1.09095	37	
Between animals having different food habits	1	0.38487	−2.80085	20.38336			
Within each type	37	0.38093	−7.94776	22.80887	0.61153	36	0.01699
For test of significance of mean respiration with effect of weight removed					0.47942	1	0.47942

Variance ratio $F = \frac{0.47942}{0.01699} = 28.2$; $F_{0.999}(1, 40) = 12.61$. Since $F(1, 36) > 12.61$, reject null hypothesis.

fitted to the data for the herbivores, *Calanus finmarchicus, C. hyperboreus, Rhincalanus nasutus,* and *Acartia clausi* (line A), and a second line fitted for the suspected carnivores, *Paraeuchaeta norvegica, Bathycalanus, Euchirella rostrata, Phronima, Euthemisto compressa, Hyperia galba,* and the euphausids (B). For the herbivores an additional regression line was also computed, omitting the data for *Acartia* since it had been acquired originally for different reasons (A′). Obviously the slope of the regression lines for the herbivores (including *Acartia* data) and the non-herbivores is not significantly different (0.348 and 0.350, respectively). With the *Acartia* data omitted the slope of the regression line fitted to the herbivore data becomes 0.441, but it can be shown statistically (Student's t test) that the slopes are still not significantly different.

To test the null hypothesis that there is no difference between the regression lines for herbivores and non-herbivores, an analysis of covariance was employed as shown in Table III. Since the slope for the herbivorous animals was not affected

by removing the *Acartia* data, it was decided to include this in the overall analysis. It is one of the advantages of covariance analysis that differences between groups of measurements can be tested in a single operation without the effect of other measurements that would normally complicate the interpretation. Thus, errors of estimate are calculated from the sums of squares and sums of products in such a way that the variance ratio tests only the respiratory rates after adjustment to remove the effect of the variable weight. Since the F test suggests that the distribution of respiration rates in relation to feeding type observed here would occur by chance much less than one time in a thousand, clearly the reason for the distribution bears some consideration.

Certain of the non-herbivorous zooplankton organisms studied were observed to be quite dense, with a hardened exoskeleton. Thus, organisms such as *Euchirella rostrata, Euthemisto compressa,* and *Hyperia galba* are presumably carrying around a considerable weight of inert organic material. More muscle protein, also quite dense, and more energy would be required to maintain the organism in the water column against its negative buoyancy.

There is little doubt that many herbivorous copepods also carry a high portion of their body weight as inert organic material, but in this case the substance may be oil with a positive buoyancy. In the case of the large *Calanus hyperboreus,* copepods of approximately the same external dimensions may differ in dry weight by several hundred per cent because of differences in the amount of stored oil.

It is also probable that a carnivorous animal is normally more active than an herbivorous one, regardless of their basal metabolic rates. The predator has to move about actively in search of the prey and then must overcome the natural reluctance of the prey to be caught by using its greater physical strength and swiftness. On the other hand, the prey organism in this association is more often than not the herbivorous copepod which can feed while it swims with a more or less continuous expenditure of a smaller amount of energy, since its food has at best extremely feeble power of escape.

Finally the possibility remains that a real difference could exist in the form of the organic matter oxidized by the herbivore and non-herbivore. Thus, an organism which burns carbohydrate exclusively would use decidedly less oxygen per unit of carbon oxidized than one which metabolizes oxygen-poor fats.

Before leaving the subject of respiration, a few remarks should be made concerning *Pleuromamma robusta* and its somewhat enigmatic position metabolically. As remarked earlier, it would appear that this species belongs in the herbivorous group, but so far as its respiration is concerned the organism would seem to be more closely allied to the non-herbivores. Clearly, with only two points on the graph it is not certain that any real difference exists between this and other herbivorous forms so far as respiration is concerned, and yet it remains possible that the very high metabolic rate observed for this form may be in some way related to the fact that it has a powerful bioluminescent organ. The species should certainly be given further attention.

Discussion

There is a persistent tendency for the biologist working with the most complex of organized systems to seek a simple solution or approximation which holds for the majority of cases. Thus certain scientific "laws" as $Q_{10} = 2$, metabolism equals

a constant times weight to some power between 0.7 and 0.8, weight equals cube of length, and so on have come into common usage in environmental studies. Most of these "laws" had their origin in laboratory studies on mammals and they need re-examination before being applied to many ecological situations. It would be a great advantage to the biological oceanographer to have a single expression which would predict the metabolism of a population of organisms with a given size distribution under any temperature conditions, but as more data become available the evidence would suggest that such a prediction equation, if it is ever formulated, will give a high-speed computer a good workout.

The relationship between metabolism and size in the warm-blooded organisms on the average has the form

$$M = 70W^{0.74} \text{ cal./day}, \tag{5}$$

but as Kleiber (1947) admits, the basis for this relationship is not really understood for the best known group of mammals. In the case of warm-blooded organisms metabolism is usually measured at or near the same temperature and the necessity to maintain this temperature against external environmental temperatures often very different would seem to give some importance to the relative surface area across which heat would be lost or gained.

In the sea, among the cold-blooded organisms there is tremendous variety in body form, biochemical mechanism and chemical composition. The surface of the marine organism is important for several reasons. Besides heat, nutrient substances, excretory substances, and gases pass back and forth across body membranes, and the frictional resistance of the surface area with the surrounding medium is critical for all organisms which move from place to place, whether they swim or are carried by their environment. It would seem very coincidental indeed should all the myriad forms of life be governed by a law derived from the warm-blooded organisms.

Among the marine invertebrates these problems have been examined in detail only for the Crustacea, and here it is true that there are relationships between metabolism and size very similar to those derived for homeothermal organisms. However, the effect of environmental temperatures on these relationships has not been given much prior consideration.

That other factors besides size and the direct effect of temperature influence respiratory rates of zooplankton forms has also been demonstrated recently. Conover (1956) observed a possible seasonal adaptation to the changing temperature regime for *Acartia clausi* and *A. tonsa*. Subsequently Conover (1959) and Marshall and Orr (1958) demonstrated seasonal differences in the respiration rate of several neritic species. Conover (1959) also demonstrated a significant difference between the respiratory rate of *Acartia clausi* in Long Island Sound and in Southampton Water. It can be seen from Table I of this study that *Calanus finmarchicus* had a decidedly higher respiratory rate on Georges Bank than in the Gulf of Maine. In this case, the populations are separated by not more than a hundred miles of water. To this list can now be added the difference in metabolism between organisms belonging to the same community but differing in their position within the food chain.

As a final note of complexity to the already confused picture of metabolic rela-

tionships for the planktonic organisms, it must be emphasized that even though reliable data were available for respiration per unit mass for a group of organisms, conversion of this information to food requirements or energy flow might introduce an appreciable error, due to insufficient knowledge regarding the nature of the food substance oxidized. It is well known that an animal requires more oxygen to oxidize fat than to oxidize carbohydrate. Although more energy is produced per volume of oxygen used in the case of carbohydrate metabolism, there is also more energy available per gram of fat than per gram of carbohydrate. Raymont and Conover (unpublished data) observed that several zooplankton organisms oxidized carbon in some form at a rate of at least ten times greater than changes in their carbohydrate reserves would predict. For instance, in the case of *Calanus hyperboreus*, the respiration rate was equivalent to an oxidation of 10 to 20 μg. of carbohydrate per day, an amount somewhat greater than the total carbohydrate content of the animals, and yet there was no detectable change in the total sugars.

It is becoming increasingly popular among environmental biologists to think of production as a single dynamic process which can be made to conform to some idealized mathematical model. Such an approach has had useful application, for instance in the North Sea pelagic fishery studies by Cushing (1955, 1959). Yet, as Steele (1960) points out, herring eat *Calanus* but they do not eat salps, even though each has a similar position in the food chain. Perhaps it is fortunate that salps are not particularly abundant in the North Sea! In any event, there would seem to be a good argument in favor of an increased emphasis on certain qualitative aspects of energy dynamics and food relations in the marine environment as a supplement to the purely quantitative approach.

Summary

1. The respiration rate of twelve species of zooplankton, the majority from oceanic waters and from depths greater than 100 meters, has been measured at temperatures close to that of their environment (4–8° C.). In most cases healthy specimens were brought to the laboratory and their food habits and behavior studied.

2. The following species seemed largely herbivorous: *Calanus finmarchicus, C. hyperboreus,* and *Rhincalanus nasutus.*

3. The copepod *Paraeuchaeta norvegica,* and the amphipods *Euthemisto compressa* and *Hyperia galba* all took animal food readily. *Bathycalanus* sp., *Euchirella rostrata* and the euphausids also are believed to be at least partially carnivorous although they demonstrated little or no feeding.

4. The animals studied had a total range in dry weight of nearly four orders of magnitude. When log respiration was correlated with log weight, a positive linear regression coefficient of 0.65 was obtained. This value, which is lower than most previously determined regression coefficients relating size and metabolism in the Crustacea, may result from the lower temperatures used in these experiments compared with those used in the earlier work.

5. As confirmatory evidence, size and metabolism were related by a coefficient of 0.67 at 5° and 0.93 at 13° in the case of *Artemia salina.*

6. Those zooplankton animals which seemed to be largely carnivorous on the basis of the behavioral studies had a significantly higher respiratory rate than those which seemed to be predominantly herbivorous.

7. Some of the possible explanations and ecological implications of the above-mentioned observations are discussed.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. IV. ON ISOTOPIC EQUILIBRIUM EXCHANGES OF CALCIUM BETWEEN CORALLUM AND ENVIRONMENT IN LIVING AND DEAD REEF-BUILDING CORALS

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The experiments reported here were carried out to determine the extent to which the polyp layer covering the outside of a coral colony can act as a barrier cutting down or preventing altogether the equilibrium exchange of calcium between the corallum and the environment.

The occurrence of a calcium equilibrium exchange process between the skeletal mineral of corals and the sea water was described in a previous paper (Goreau, 1959a), but in comparing the calcium-45 uptake of living corals with that of dead macerated pieces of corallum, we observed that the incorporation of the radioactive calcium by exchange was in most cases much slower than that deposited by active secretion in living corals.

The total rate of exchange between the solid and dissolved phases in a system at equilibrium depends on the temperature and on the surface area of the solid phase in free contact with the medium. The former variable will not be considered further in this study since the temperature of most West Indian coral reefs remains stable within the range of 25° C. to 29° C. On the other hand, the total surface area that is potentially available for exchange in an average coral colony is enormous, due to the fact that the corrallum is not a solid mass of mineral matter, but a complex framework of interlocking calcareous lamellae with water-filled spaces in between. In the Scleractinia, the mineral structure is composed of more or less trabeculate spherulitic aggregates of aragonite crystals (Wells, 1956), with the living polyps located only on the external surface of the skeletal mass. In eight Indo-Pacific reef-building corals Odum and Odum (1955) estimated that the pores occupied between 7% and 38% of the total skeletal volume. Consequently, while the internal surface area of the coral skeleton must be very large it is questionable whether more than a very small fraction of this can freely exchange with the surrounding environment, since most of the outer surface of living corals is covered with a continuous sheet of polyps which tends to isolate the skeletal mass from direct contact with the sea water. Exposure of the skeleton to the medium can occur only where the polyparium has died and disappeared, but secondary encrusting organisms rapidly overgrow these parts of the colonies with a thick layer of their own.

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The water occluded in the internal pore spaces of the corallum is not in free communication with the external medium and exchanges only very slightly with it. This is demonstrated by our observations that the pore water inside of living corals immersed in sea water enriched with calcium-45 for 12 to 24 hours contains virtually no radioactivity (Goreau, unpublished). The huge internal surface area of the crystalline matrix of corals is effectively barred from exchanging with the environment by the paucity of its connections with the external medium, which means that the internal environment of a living coral head is relatively isolated from the outside.

The surface waters of the tropics are saturated with respect to aragonitic and calcitic $CaCO_3$. At the temperatures and salinities normally encountered in a coral reef, the solubility product of aragonite is quite large, about 10^{-6} (Revelle and Fairbridge, 1957). Hence, the corallum, which is composed of nearly pure aragonite (Meigen, 1903; Lowenstam, 1954; Chave, 1954), will undergo chemical exchange with the aquatic environment according to the reaction

$$CaCO_{3\,(s)} \rightleftharpoons CaCO_{3\,(l)} \rightleftharpoons Ca^{++} + CO_3^{--}, \text{ etc.}$$

No net deposition or solution can occur as long as the system is in chemical equilibrium, but when the solid phase is enriched in calcium-45 and is in contact with freely flowing sea water saturated with $CaCO_3$ containing no calcium-45, then isotopic equilibrium cannot be established and the radioactive calcium will be lost from the coral at a rate directly proportional to the rate of the chemical equilibrium exchange. This condition was produced by "infecting" living corals with calcium-45 and then measuring the rate at which the isotope was lost when the living colonies were placed back on the reef and allowed to resume growth under natural conditions for a prolonged period of time.

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Loss of Calcium by Exchange from Living and Dead Corals

The observations cited in this section were carried out on colonies of *Porites furcata* (Lamarck) and *Millepora alcicornis* (Linnaeus). The latter is not a scleractinian coral but a hydrocoral belonging to the order Milleporina of the class Hydrozoa (Hyman, 1940). These organisms secrete a massive aragonitic skeleton; in the West Indies the Milleporina are important reef-builders in association with the shallow water Scleractinia (Goreau, 1959b).

For the purpose of these experiments we chose large and symmetrical colonies growing at a depth of about five feet on the windward reef at Magueyes Island, which is situated off the southwest coast of Puerto Rico. The symmetry of

these colonies indicated that they were growing at about the same overall rate in all directions. Two whole specimens of each were carefully detached from the reef and transferred into large plastic tubs without being allowed to come into contact with the air. These were ferried by boat to the nearby laboratory jetty where, still in their original containers, they were put under a twin bank of 80-watt fluorescent lights located about 18 inches above the specimens. The water was stirred and aerated by a stream of saturated air. After a 24-hour period of acclimatization neutral $Ca^{45}Cl_2$ was added to a final activity of about 0.14 μc/ml. The corals were exposed to this concentration of calcium-45 for 48 hours. For the purpose of measuring specific activity, 50-ml. water samples were withdrawn one, 24 and 48 hours following addition of the isotope. The water temperature throughout this period was $28 \pm 1.5°$ C. At the end of the run, the corals were transferred to a large wire mesh cage anchored on the reef in approximately 4½ feet of water, close to the site from which they had originally been collected and where the ecological conditions were the same. The bottom of the cage, made from one-inch galvanized iron mesh wire, was raised about 12 inches off the sea bottom by wooden legs, to protect the specimens from sediment, and at the same time expose them adequately to the prevailing water currents.

Samples were obtained by breaking off groups composed of 10 to 15 individual branches from each colony. The first samples were taken three hours after removal of the corals from the radioactive sea water. Fourteen samples of *P. furcata* were taken over a period of 23 days. *M. alcicornis* was sampled nine times over the first ten days, and twice more at 154 and 156 days.

The samples were cleaned of all worm tubes, ophiuroids, sponges, algae and other encrusting organisms, and then dried at 85° C. for one week. In the case of the *P. furcata* samples, the upper living parts of the branches covered by the dark brown polyparium were separated from the older greenish grey parts which were dead. The two series of samples were kept for separate analysis. A parallel series of dead samples was not obtained from *M. alcicornis* because the entire colonies were covered by a sheet of living polyparium.

The cleaned and dried samples were dissolved in concentrated HCl after addition of 1 ml. octyl alcohol to reduce the violent foaming. The suspension was homogenized, boiled, cooled and made up to a standard volume with double distilled water; the calcium-45 activity was determined on duplicate 1-ml. aliquots using the oxalate precipitation method described in a previous paper (Goreau, 1959a). All results were expressed in counts per minute per milligram calcium, *e.g.* the specific activity. Due to the long duration of this experiment, the activities measured were corrected for radioactive decay, using the time of removal of the corals into inactive sea water as zero time.[2]

The results of these experiments are summarized in Table I. Each datum represents the mean specific activity of a group of 10 to 15 individual branches broken off at random from the test colonies at the times indicated in the left-hand column of the table. The mean specific activity only is given for each group since the individual branches constituting each sample were pooled together.

As is shown in Figures 1A and 1B for *P. furcata*, there is considerably more scatter of our results in the samples taken from the living parts of the corals, where

[2] The half life of calcium-45 is 163 days.

most of the calcium-45 was incorporated by active deposition, than in the dead samples in which the calcium-45 content was due entirely to equilibrium exchange. Such variations, characteristic of living corals, are due to short term inequalities of the calcium deposition rates in different parts of the colonies tested, and may be evidence of a discontinuous growth process (Goreau and Goreau, 1959). Had these corals been exposed to the calcium-45 for a considerably longer period than 48 hours, the variations in the specific activity would probably have been much

TABLE I

Changes in Ca^{45} activity with time in living and dead corals. The figures are specific activities in counts min.$^{-1}$ mg. Ca^{-1}

Time in days	*Porites furcata*		*Millepora alcicornis*
	Live	Dead	
0.125	255.9	55.8	66.4
1	202.6	33.8	89.2
2	266.8	30.8	
3		35.2	54.1
4			56.3
5			45.0
7	244.4	19.1	70.1
8	274.3	25.1	50.1
9			49.9
10			52.6
12	267.2	22.7	
13	252.9	24.4	
14	265.8	18.7	
17	286.8	17.8	
18	277.3	19.5	
19	248.3	20.2	
21	234.4	14.2	
22	230.5	10.6	
23	237.2	19.5	
154			38.2
156			76.4

smaller, or even absent. From the symmetrical appearance of the test corals used, we assumed that the average growth rate was on the long run about the same in all parts of the colonies. Since these corals were exposed to calcium-45 for only two days, the pattern of random variation in the growth rates that existed during this time was essentially "frozen" when the colonies were removed into inactive sea water.

In Figure 1, the changes in activity with time are shown for *P. furcata:* curve A represents the living parts, curve B the dead branches. The regression lines drawn through the points were fitted by the method of least squares. The distribution of points in the case of the living branches of both *P. furcata* (Fig. 1A) and *M. alcicornis* (Fig. 1C) is such that an attempt to derive a general relationship for the rates of change of the specific activities with time yielded meaningless equations, and the slopes of the regression lines are too small to be significant on the basis

of the available data. This means that there was no significant loss of calcium-45 by isotopic exchange from the living parts of both species tested over the time of our observations, which was 23 days for *P. furcata* and 156 days for *M. alcicornis*.

As seen from the curve in Figure 1B, the situation was very different in the dead parts of *P. furcata* where the naked corallum was directly exposed to the circulating sea water. There is a progressive decrease of the radioactivity with time, due to loss by isotopic exchange. Because of the smaller scatter of the

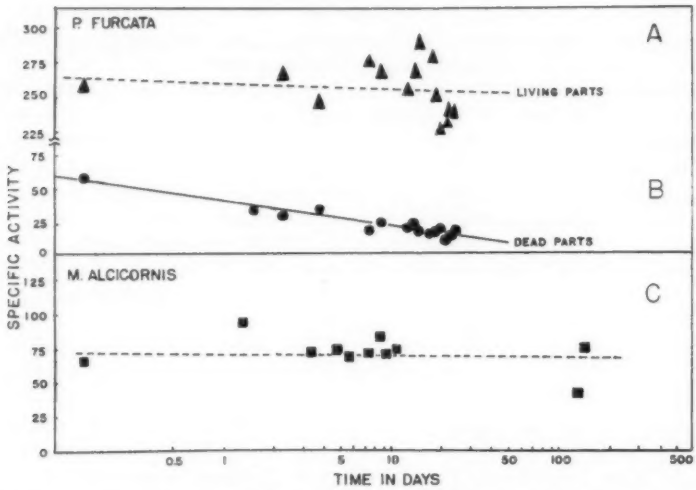

FIGURE 1. Changes of specific activity as a function of the time in the skeletons of living and dead corals, plotted on semi-logarithmic coordinates. Curves A and B represent the activity due to calcium-45 over a period of 23 days in living and dead *Porites furcata*, respectively. Curve C shows the same in *Millepora alcicornis* over a period of 156 days. The regression lines were fitted by the method of least squares. Note that no significant loss of calcium-45 activity occurs in the living corals, but in the dead parts of *Porites furcata* the slope of the curve shows that the exchangeable calcium-45 activity has a half-life of approximately five days.

data the fit of the regression curve to the points is quite good. The line yields the equation

$$A = 200\, e^{-0.136\, T}, \qquad (1)$$

for the relation between the specific activity of the corallum (A) and the time (T). The slope of the curve shows that one-half of the original activity on the surface of the dead parts of *P. furcata* colonies is lost in about five days, and nearly all in about 110 days.

CALCIUM EXCHANGE IN DEAD CORALS WITH AND WITHOUT COENOSARC

The effect of the mechanical presence of a tissue barrier on the calcium exchange in corals was tested by comparing isotopic equilibration rates in killed corals, in

which the coenosarc was still present, with that in corals from which the fleshy parts had been removed by maceration.

Corals of several different species were killed and preserved in sea water containing 4% formaldehyde buffered to pH 8 with bicarbonate. Each specimen was split into two pieces, one being preserved in the formaldehyde while the other was macerated in sea water until the tissue layer covering the outside of the corallum was completely removed. The clean macerated halves were then placed together with the preserved halves into the buffered sea water-formaldehyde solution, and neutralized $Ca^{45}Cl_2$ was added to a final activity of approximately 0.05 μc./ml. The medium was stirred with a stream of saturated air pumped through an airstone. The corals were allowed to soak in the radioactive sea water for 48 hours, the temperature being maintained at $28 \pm 1°$ C. during this time. Samples of water were withdrawn for measurement of the specific activity at the beginning and at the end of the runs. The pieces of coral were drained on filter paper to remove the most of the adherent radioactive water, rinsed in six changes of large volumes of inactive sea water over a period of about 15 minutes and allowed to drain for eight hours on blotting paper. All specimens were treated exactly the same way.

TABLE II

Exchange uptake of Ca^{45} by killed corals with and without coenosarc. Average specific activities in counts $min.^{-1}$ mg. $Ca^{-1} \pm$ S. D. Number of colonies sampled in brackets

Species	Macerated colonies without coenosarc	Colonies with coenosarc
Acropora cervicornia	11.20 ± 1.00 (4)	10.10 ± 0.80 (4)
Manicina areolata	0.82 ± 0.10 (5)	1.78 ± 0.10 (5)
Montastrea annularis	1.70 ± 0.20 (4)	1.70 ± 0.10 (4)
Montastrea cavernosa	1.00 ± 0.20 (4)	1.10 ± 0.05 (4)
Diploria strigosa	0.60 ± 0.05 (4)	1.50 ± 0.06 (4)

Sampling was carried out by cutting off 3-cm.-long pieces from the terminal parts of branching corals, or taking core samples with a hollow punch from the massive corals (*cf.* Goreau and Goreau, 1959). To make the data from macerated and unmacerated specimens as comparable as possible, great care was taken to sample only strictly corresponding areas of the colonies. Three pieces were removed from each test specimen. These were once more rinsed in fresh sea water for 30 seconds, drained on filter paper, and dissolved in individual flasks containing concentrated HCl. The samples were homogenized, boiled, cooled, made up to a standard volume and the radioactivity determined by the oxalate method on replicate 1-ml. aliquots. Due to the low activities of the same samples, the time taken for 2000 counts was used as a basis for our measurements, and the results expressed as counts per minute per milligram calcium.

The results of these experiments are given in Table II for five species of reef-building corals. The numbers represent the specific activities; each value is the mean of three measurements. The numbers in brackets correspond to the number of separate colonies tested.

In three of the five species tested, there were no significant differences in the amount of calcium-45 taken up by the macerated specimens as compared with those still having a mechanically intact layer of tissue. The brain corals, *Diploria strigosa*

and *Manicina areolata,* were the exceptions to this; significantly higher specific activities were observed in the preserved colonies which still had the intact coenosarc. As there is no evidence that coral tissues contain substances which bind inorganic calcium, we believe that the increased activity was due to occlusion in the coral of a small amount of radioactive sea water that was not washed out completely. The rinsing procedure was kept as short as possible to prevent loss of calcium-45 from the specimens by exchange with the wash water.

These data indicate that the mere mechanical presence of an intact, but dead, tissue layer does not appear to hinder significantly the isotopic exchange of calcium between the corallum and medium over a period of 48 hours.

A notable feature of these results is that much more calcium-45 was exchanged by the skeleton of *Acropora palmata* than by any of the other corals tested. This is due to the greater porosity and surface area in the corallum of the Acroporidae as compared with that of the Faviidae, the family to which all the rest of the corals listed in Table II belong. The isotope exchange method may eventually become useful as a quantitative measure of the skeletal surface area and porosity in different corals if suitable reference standards can be developed.

Calcium Exchange in Corals With Extremely Low Growth Rate

The experiments described in this section were conducted on colonies of *Oculina diffusa* in which the active calcium uptake rate was depressed to extremely low levels through the combined removal of the zooxanthellae and treatment with acetazolamide (2-acetylamino-1,3,4-thiazole-5-sulfonamide) to inhibit the enzyme carbonic anhydrase (*cf.* Goreau, 1959a). The calcium-45 deposited by these corals was compared with that taken up through isotopic exchange by control colonies of the same species which had first been killed and macerated to remove the tissue layer covering the skeleton.

Zooxanthellae were removed from the corals by keeping the colonies in darkened aquaria for two months. The corals fed on zooplankton brought in from the outside by the circulating fresh sea water supply showed no signs of starvation from this treatment. Complete extrusion of the zooxanthellae caused the coenosarc to change from a translucent yellowish brown to a transparent colorless appearance. The "bleached" corals were healthy and fully expanded at all times, but their calcium deposition rates were extremely low in comparison with normal colonies of the same species containing a full complement of zooxanthellae (Goreau, 1959a; Goreau and Goreau, 1959, 1960).

The zooxanthella-less corals and the macerated controls were distributed into four glass jars, two of which were painted black to exclude light. One light-dark pair contained only fresh sea water, the other contained sea water with 10^{-3} *M* acetazolamide.[3] For the 48-hour duration of the experiment, the jars were kept under a twin bank of 80-watt fluorescent tubes, the temperature was maintained at $28 \pm 0.5°$ C. and the contents of the jars were stirred and aerated with a fine stream of saturated air.

After the corals were exposed to the action of 10^{-3} *M* acetazolamide for 24

[3] Kindly supplied as "Diamox" by the Lederle Laboratories Division of the American Cyanamid Company.

hours, neutral $Ca^{45}Cl_2$ was added to a final concentration of about 0.1 μc./ml. Multiple samples of the living experimental colonies and the dead controls were taken at 3-, 7-, 12- and 15-hour intervals after addition of the isotope. Water aliquots were taken at the same times to measure the specific activity of the medium. The coral samples, taken by breaking off terminal portions of the branches, were immediately drained on filter paper, rinsed in six changes of fresh inactive sea water, drained again and dissolved in dilute HCl (1 : 1) in individual volumetric tubes. Analysis for calcium-45 activity was done by the oxalate method and the results calculated in terms of the specific activity, *e.g.* counts per minute per milligram calcium. At the end of the experiment the remaining corals which had been in the acetazolamide solution for a total of nearly 40 hours appeared as healthy as the controls kept in plain sea water. The colonies were fully expanded and able to ingest small pieces of clam meat. When returned to fresh sea water, they lived for a further two weeks until sacrificed for histological purposes.

TABLE III

Calcium-45 activities in living zooxanthella-less Oculina diffusa compared with dead control colonies, in light and darkness, and in presence or absence of acetazolamide. Figures represent the specific activities in counts min.$^{-1}$ mg. Ca^{-1} ± S.D. Number of samples in brackets

Time in hours	Plain sea water			
	Light		Dark	
	Living corals	Dead corals	Living corals	Dead corals
3	1.9 ± 0.1 (4)	2.6 ± 0.3 (3)	1.4 ± 0.2 (4)	1.6 ± 0.2 (3)
7	5.3 ± 1.0 (5)	3.5 ± 0.2 (3)	3.0 ± 0.8 (3)	2.7 ± 0.1 (3)
12	9.4 ± 0.6 (3)	3.6 ± 0.2 (3)	3.1 ± 0.3 (5)	4.0 ± 0.1 (3)
15			3.8 ± 0.5 (4)	4.2 ± 0.05 (3)
	10^{-3} *M* acetazolamide			
3	1.4 ± 0.1 (4)	1.6 ± 0.2 (3)	1.2 ± 0.01 (4)	3.7 ± 0.1 (3)
7	0.95 ± 0.3 (6)	3.0 ± 0.3 (3)	1.4 ± 0.1 (4)	4.4 ± 0.1 (3)
12	1.9 ± 0.6 (5)	3.3 ± 0.05 (3)	1.8 ± 0.2 (3)	5.0 ± 0.05 (3)
15	1.3 ± 0.6 (4)	3.8 ± 0.2 (3)	2.0 ± 0.5 (4)	4.9 ± 0.2 (3)

The results of these experiments are given in Table III. In the absence of acetazolamide, the zooxanthella-less corals which had been kept in darkness (Fig. 2B) contained about the same specific activity as the dead controls, whereas those growing in light had more than twice the activity of the controls (Fig. 2A). The reason for this is not clear since zooxanthellae appeared to be completely absent from these colonies. Some potentiation of the growth rate might have occurred if boring algae were still present, but no chlorophyll tests were made on these specimens at the time. In previous experiments on this and other corals from which zooxanthellae had been removed, we found no significant differences of the calcium uptake rates in light and darkness (Goreau, 1959a).

In the presence of 10^{-3} *M* acetazolamide, the calcification rate of the experimental colonies was so reduced that they contained less than half of the specific activity of the dead controls. The effect was the same in light as in darkness (Figs. 2C and 2D). Whereas the activity of the dead controls increased continuously for the first 12 hours, the activity of the living test corals showed little

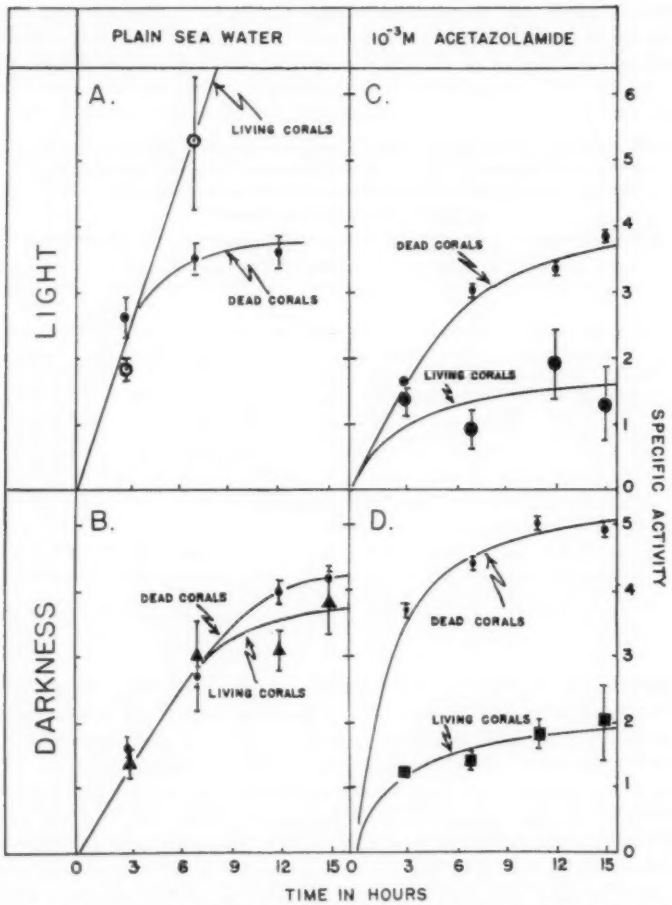

FIGURE 2. The effect of the inhibition of carbonic anhydrase by 10^{-3} *M* acetazolamide on the rate of calcium-45 incorporation by zooxanthella-less *Oculina diffusa*, compared with the rate of isotopic exchange in dead macerated control colonies, in light and in darkness. Calcium-45 incorporation by living and dead corals in plain sea water is shown in Figures 2A and 2B. Figures 2C and 2D show the same in the presence of 10^{-3} *M* acetazolamide. Note that in the presence of the carbonic anhydrase inhibitor the specific activity of the living corals is less than half that of the dead exchange controls.

increase beyond that incorporated during the initial three hours of the experiment. We consider it probable that most of this activity was due to a small amount of calcium taken up into the tissues but not deposited into the skeleton. It is clear from these results that the living zooxanthella-less colonies in which the enzyme carbonic anhydrase was also inhibited probably did not grow at all. However, due to the presence of the living coenosarc, the process of isotopic exchange between the skeleton and medium was very much slower than in the dead controls in which the skeletal surface was in direct contact with the medium and so free to exchange with it.

Discussion

The equilibrium exchange rate of calcium between the coral skeleton and the surrounding sea water is very different in dead corals as compared to living corals. We have found earlier that isotopic equilibration between corallum and sea water enriched with calcium-45 is more or less complete in about 48 hours at a temperature of 28° C. (Goreau, 1959a). However, corals in which the calcification rate was reduced to very low levels by the simultaneous removal of zooxanthellae and inhibition of carbonic anhydrase with acetazolamide contained less radioactivity than macerated "exchange controls." Our data indicate that the mechanical presence of the coenosarc does not hinder calcium equilibrium exchange if the tissues have been killed. On the other hand, the living coenosarc is comparatively calcium-proof, thus preventing the underlying skeletal mass from exchanging with the surrounding environment. This is supported by our previous observation that the living polyps of *Astrangia danae*, a cold-water coral, tend to exclude calcium relative to the sea water in which they are growing (Goreau and Bowen, 1955).

In corals growing under natural conditions on a reef the exchange rate of calcium between the skeleton and the medium is much higher in the dead parts of the colonies which are unprotected by the polyparium than in those parts which are covered by living coenosarc. The extremely low rate of exchange in this case is not entirely due to the isolating action of the living tissue sheet covering the skeleton: after the colonies were labelled with calcium-45 they were returned to the sea where they continued to grow, so that the radioactive layer eventually became buried under successive new layers of inactive skeletal material. Under these conditions the radioactive material was well protected from isotopic exchange which can occur only at the skeletal surface, in consequence of which no significant loss of calcium-45 could be observed.

The low exchangeability of already deposited calcium indicates that it does not significantly take part in physiological interactions with the overlying coral tissue. This is very different from what is observed in vertebrates where 25% to 35% of the bone calcium remains exchangeable with the calcium dissolved in the medium (*cf.*, for example, Dawson, 1955). The much greater exchangeability of the skeletal calcium in the vertebrates as compared with that of the corals is due to the fact that the bones of the former constitute an endogenous tissue system in dynamic equilibrium with the rest of the body constituents (Armstrong, 1955; Neuman and Neuman, 1953; Marshall, 1960), whereas the scleractinian corallum is an exoskeleton which, once formed, is entirely outside of the body proper, and its mineral contituents do not appear to be in steady-state equilibrium with the "milieu intérieur" of the polyps.

Summary and Conclusions

1. Equilibrium exchange of calcium between the skeleton and the medium was measured in dead and living corals under various conditions, using calcium-45 as isotopic tracer.

2. The calcium which is deposited as aragonitic carbonate into the skeleton of living corals is not in contact with the environment. Under the conditions of our experiments, no significant isotopic calcium exchange occurred in *Porites furcata* and *Millepora alcicornis* over a period of 23 days and 156 days, respectively.

3. In the dead parts of *Porites furcata* colonies where the corallum was exposed to direct contact with the medium, rapid isotopic exchange occurred so that the half-life of calcium-45 activity on the skeletal surface was only about five days.

4. In reef corals killed with formaldehyde, the amount of calcium-45 activity picked up by the skeleton from the medium in 48 hours by equilibrium exchange was the same in colonies in which the coenosarc had been preserved as in those from which all tissues had been removed, so that the naked corallum was in direct contact with the radioactive sea water. This indicates that, under the conditions of our experiments, the mechanical presence of the non-living coenosarc did not constitute a barrier to equilibrium exchange between the corallum and the environment.

5. In living corals in which the calcification rate was depressed to extremely low levels by simultaneous removal of the zooxanthellae and inhibition of carbonic anhydrase with 10^{-3} *M* acetazolamide, the amount of calcium-45 activity incorporated was 50% to 70% less than that exchanged by the dead control colonies from which all tissues had been removed by maceration. This indicates that the *living* coenosarc is an effective barrier to equilibrium exchange of calcium between the skeleton and the environment, even when the calcium deposition rate is almost zero.

6. Our experimental evidence indicates that the living coenosarc is fairly calcium-proof and prevents the skeleton from exchanging with the environment. Unlike vertebrates, in which a large fraction of the bone calcium remains in dynamic equilibrium with the dissolved calcium of the body fluids, the skeletal calcium of corals, once it is deposited into the corallum, probably does not take part in steady-state or equilibrium exchanges with the overlying tissues.

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RESPIRATORY REGULATION IN AMPHIBIAN DEVELOPMENT [1]

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There is now considerable evidence (reviewed by Slater and Hülsmann, 1959) that the respiratory rate of a cell abundantly supplied with oxidizable substrates is a function of the rate at which it metabolizes labile phosphorus compounds in response to energy demand. Consider, for example, the oxidation of glucose:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O.$$

Normally, this process is coupled to the esterification of inorganic phosphorus:

$$C_6H_{12}O_6 + 6\,O_2 + 38\,ADP + 38\,H_3PO_4 \rightarrow 6\,CO_2 + 44\,H_2O + 38\,ATP.$$

When work is to be done, stored ATP is utilized:

$$38\,ATP + 38\,H_2O \rightarrow 38\,ADP + 38\,H_3PO_4 + Energy.$$

As a result, the levels of ADP and inorganic phosphorus are raised momentarily, and respiration is quickened until ATP is restored. Thus, under normal conditions, the respiratory rate of a cell depends upon its rate of energy expenditure.

In the presence of uncoupling agents, *e.g.*, 2,4-dinitrophenol (DNP), the link between oxidation and phosphorylation is severed. When this happens, respiration proceeds at a rate limited only by the availability of oxidizable substrates, and without concurrent formation of ATP. At the same time, ATP-stores are depleted by destructive catalysis, and the ability to perform work deteriorates rapidly.

The burgeoning respiratory rates of developing embryos surely reflect ever-increasing expenditures of energy. Therefore, a study of the respiratory responses of developing embryos to uncoupling agents should yield important information about the energetics of development. Along these lines, a recent study of sea urchin embryos by Immers and Runnström (1960) has provided interesting data, and there is reason to believe that amphibian embryos are amenable to similar analysis. In the presence of DNP, explants of the tissues of frog gastrulae respire at twice the normal rate (Ornstein and Gregg, 1952); and, under similar conditions, intact gastrulae are partially depleted of their stores of esterified phosphorus and are prevented from undergoing further morphological change (Gregg and Kahlbrock, 1957).

In the work about to be reported, an uncoupling agent (DNP) has been used to study the development of respiratory regulation in *Rana pipiens* embryos, and also in hybrid embryos obtained by fertilizing *Rana pipiens* eggs with *Rana sylvatica* sperm. First studied by Moore (1946), these hybrids are incapable of developing

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beyond the early gastrula stage, and exhibit numerous other morphogenetic or metabolic anomalies (Gregg, 1957).

Methods

Embryological

Developing embryos were obtained by stripping eggs from pituitary-injected *Rana pipiens* females into suspensions of active *Rana pipiens* or *Rana sylvatica* sperm. After two hours, they were dispersed thinly among fingerbowls and allowed to develop at temperatures ranging from 10° C. to 25° C. The medium, 10% Ringer's solution without phosphate or bicarbonate, was changed every two days, or more often. Before manometric measurements were made, watchmaker's forceps were used to free the embryos of their jelly coats.

Manometric

Respiratory rates were determined with a refrigerated Warburg respirometer equipped with 7-ml. single-side-arm center-well flasks. Carbon dioxide was absorbed on filter paper rolls placed in the center wells and saturated with 10% KOH. The flasks were shaken 75 complete cycles per minute at an amplitude of 6 cm. The temperature of the water bath was held constant at 24° C. Further details will be cited as the need arises.

Terminological

Developmental stages were determined by reference to the charts of Shumway (1940), which standardize the course of *Rana pipiens* development at 18° C. Therefore, regardless of their actual temperature histories, embryos in a given Shumway stage have been assigned the corresponding standard age at 18° C. Hybrid embryos have been assigned the same stages, and ages, as simultaneously developing *Rana pipiens* controls.

Respiratory rates are expressed in the following units: microliters of oxygen per hour per 50 embryos.

The respiratory rate exhibited by intact embryos at a given stage, and under standard conditions, is called the *respiratory norm* of embryos at that stage.

The respiratory rate exhibited by intact embryos at a given stage, and under maximal stimulation by DNP, is called the *respiratory potential* of embryos at that stage.

The quotient obtained by dividing the respiratory potential by the corresponding respiratory norm is called the *respiratory control quotient.*

Results

The results obtained in the present work are now listed without commentary. They will be discussed in the next section.

(1) The respiratory norm of *Rana pipiens* embryos is a strongly increasing function of developmental age (Table I, Table III).

(2) The relation between the developmental age and respiratory norm of *Rana pipiens* embryos is best characterized as the exponential function consisting of all pairs (t, y) satisfying the following equations:

(i)
$$y = 5\,e^{0.026t} \qquad (0 \leqslant t \leqslant 56)$$
$$y = 21\,e^{0.017\,(t-56)} \qquad (56 \leqslant t \leqslant 140)$$

TABLE I

Influence of 2,4-dinitrophenol on the respiratory rate of Rana pipiens embryos

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar					
			Control	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}	10^{-1}
A	11	34	11	19	24	33	31	—
	12½	46	18	28	35	40	35	1
	16	72	29	42	50	45	39	4
	19	118	56	93	104	85	79	25
B	6	7	6	6	13	24	23	3
	10½	30	12	16	22	32	28	2
	15	67	24	33	36	40	35	9
	10½	90	38	54	71	53	51	6
	19	118	56	85	102	71	64	26
	20	140	84	112	118	104	78	19

Main compartment of each flask: 20–50 embryos in 1 ml. 10% Ringer's. Side-arm of each flask: 0.5 ml. DNP in 10% Ringer's adjusted to give final concentration shown. Side-arm contents delivered to main compartment immediately after first reading. Readings were made for four or five hours (usually five) at half-hour intervals. Respiratory rates were constant after first hour. Entries designate average rates for last three (four) hours of four (five) hour runs.

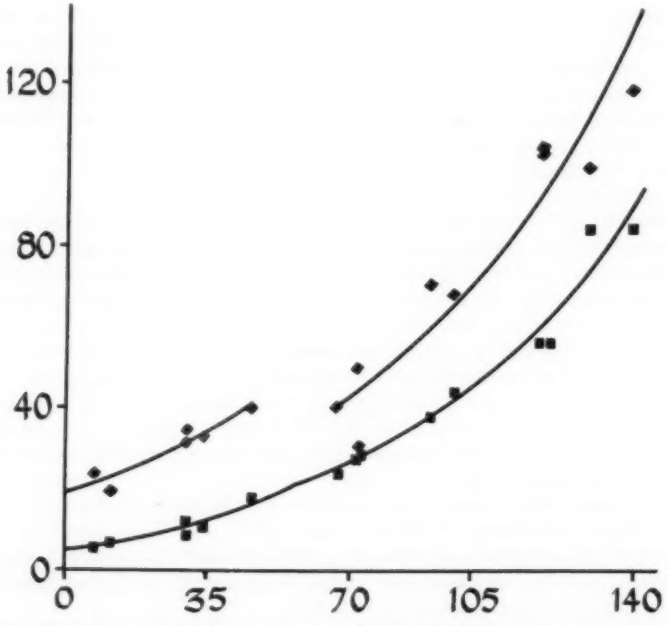

FIGURE 1. Respiratory norm (lower curve) and potential (upper curve) of *Rana pipiens* embryos. Abscissa, developmental age. Ordinate, respiratory rate.

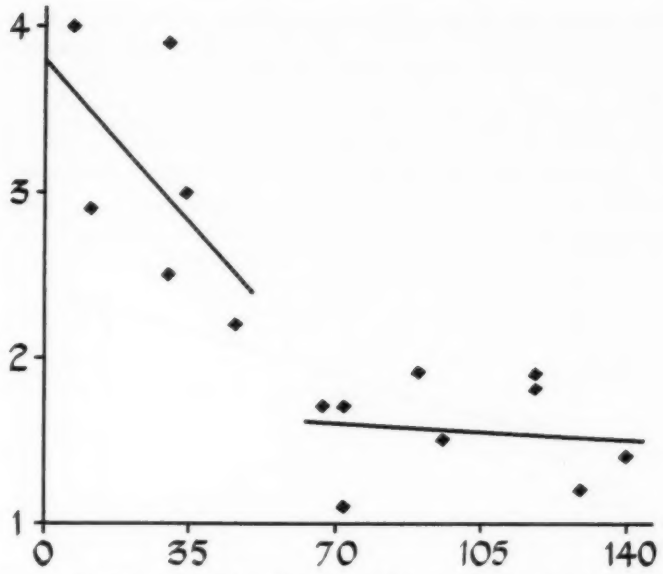

FIGURE 2. Respiratory control quotient, *Rana pipiens*. Abscissa, developmental age. Ordinate, respiratory control quotient.

TABLE II

Influence of 2,4-dinitrophenol on the respiratory rate of Rana pipiens ♀ × Rana sylvatica ♂ embryos

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar					
			Control	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}
C	7½	11	5	13	20	22	18	20
	10½	30	3	10	18	31	30	17
	13½	56	3	13	21	28	26	15
	17½	90	12	18	22	29	—	20
	19	118	11	19	21	35	32	32
D	7+	9	8	13	20	26	28	15
	11½	38	9	18	25	30	28	12
	15	67	13	17	22	35	37	19
	18	96	10	18	26	38	38	28

Fifty embryos per flask. DNP-treated embryos were equilibrated in DNP-solutions in 10% Ringer's for two hours preceding measurements. Readings were taken for one hour at 5-minute intervals.

Hybrid embryos do not develop beyond Stage 10: entries in the stage and age columns designate average developmental stages and corresponding ages of *Rana pipiens* control embryos.

where t is the developmental age and y is the respiratory norm (Fig. 1, lower curve).

(3) The respiratory activity of *Rana pipiens* embryos at any stage is stimulated by DNP in concentrations ranging from 5×10^{-6} M to 1×10^{-4} M (Table I, Table III).

(4) The respiratory potential of *Rana pipiens* pre-neurulae is exhibited under treatment with DNP at concentrations near 5×10^{-5} M; that of neurulae and older embryos is exhibited under treatment with DNP at concentrations near 10^{-5} M (Table I, Table III).

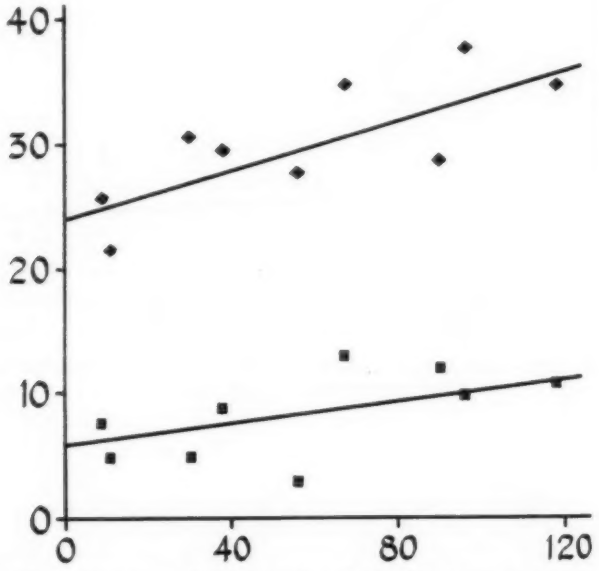

FIGURE 3. Respiratory norm (lower curve) and potential (upper curve) of *Rana pipiens* ♀ × *Rana sylvatica* ♂ embryos. Abscissa, developmental age. Ordinate, respiratory rate. (24° C.)

(5) The relation between the developmental age and respiratory potential of *Rana pipiens* embryos is best characterized as the exponential function consisting of all pairs (t, y) satisfying the following equations:

(ii)
$$y = 19\, e^{0.017t} \qquad (0 \leqslant t \leqslant 46)$$
$$y = 41\, e^{0.016\,(t-67)} \qquad (67 \leqslant t \leqslant 140)$$

where t is the developmental age and y is the respiratory potential (Fig. 1, upper curve). For reasons explained later, the respiratory potentials corresponding to some values of t $(46 < t < 67)$ are left undefined.

(6) The respiratory control quotient of *Rana pipiens* embryos decreases rapidly from 3.8 at fertilization to 2.5 at 46 hours, and slowly from 1.6 at 67 hours to 1.5 at 140 hours (Fig. 2). For reasons explained later, the respiratory control quotients of embryos between the ages of 46 hours and 67 hours are left undefined.

(7) The respiratory norm of hybrid embryos is a weakly increasing function of developmental age (Table II).

(8) The relation between the developmental age and respiratory norm of hybrid embryos is best characterized as the linear function consisting of all pairs (t, y) satisfying the following equation:

(iii) $$y = 6 + 0.045t \qquad (0 \leqslant t \leqslant 118)$$

where t is the developmental age and y is the respiratory norm (Fig. 3, lower curve).

(9) The respiratory activity of hybrid embryos at any stage is stimulated by DNP in concentrations ranging from 5×10^{-6} M to 5×10^{-4} M (Table II).

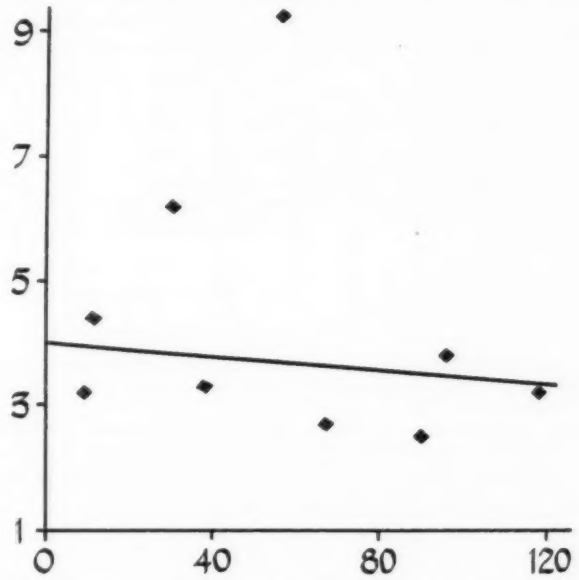

FIGURE 4. Respiratory control quotient, *Rana pipiens* ♀ × *Rana sylvatica* ♂. Abscissa, developmental age. Ordinate, respiratory control quotient.

(10) The respiratory potential of hybrid embryos is exhibited under treatment with DNP at concentrations near 5×10^{-5} M (Table II).

(11) The relation between the developmental age and respiratory potential of hybrid embryos is best characterized as the linear function consisting of all pairs (t, y) satisfying the following equation:

(iv) $$y = 24 + 0.10t \qquad (0 \leqslant t \leqslant 118)$$

where t is the developmental age and y is the respiratory potential (Fig. 3, upper curve).

(12) The respiratory control quotient of hybrid embryos decreases from 4 at fertilization to 3.3 at 118 hours (Fig. 4).

(13) Homogenization in buffer-saline solution has little effect upon the respiratory activity of young *Rana pipiens* embryos, but it powerfully stimulates the respiratory activity of older ones (Table III).

(14) The respiratory rates exhibited by homogenized *Rana pipiens* embryos are not affected by DNP in concentrations ranging from 10^{-5} *M* to 5×10^{-5} *M* (Table III).

TABLE III

Influence of 2,4-dinitrophenol on the respiratory rate of intact embryos and cell-free homogenates (Rana pipiens)

Egg clutch	Stage	Age 18° C.	DNP-concentration, molar		
			Control	10^{-5}	5×10^{-5}
E Intact	7½	11	7	—	20
	10½	30	9	—	35
	16	72	28	31	—
	18	96	44	68	—
	19½	129	84	99	—
E Homogenized	7½	11	8	6	9
	10½	30	8	10	7
	16	72	132	130	129
	18	96	132	128	124
	19½	129	146	136	137

Intact embryos. Fifty embryos per flask. DNP-treated embryos were equilibrated in DNP solutions (in 10% Ringer's) for one to two hours preceding measurements. Readings were taken for one hour at 5-minute intervals.

Homogenates. Embryos suspended in ice-cold 10^{-2} *M* phosphate buffer made up in 0.065% NaCl (50 embryos per ml.) were homogenized with a Lourdes homogenizer. Aliquots (1 ml.) were transferred to respirometer flasks. Immediately after first reading, DNP solutions (0.5 ml. in buffer-saline, "pH 7.48") were tipped into main compartments to give final DNP-concentrations desired. Readings were taken for one hour at 5-minute intervals.

Entries in the stage and age columns designate average developmental stages and corresponding ages.

DISCUSSION

The results listed in the previous section will now be discussed, in sequence.

(1) Embryologists have known for a long time that developing amphibian embryos are characterized by waxing respiratory norms. Our first result, then, is not new, and our chief concern will be to find an explanation for it.

One explanation is provided by the assumption that developmentally increasing respiratory rate is the direct result of the synthesis of respiratory machinery. But recent experiments (Spiegelman and Steinbach, 1945; Gregg and Ray, 1957) have established that homogenates of newly fertilized eggs can be made to respire endogenous substrates at rates exceeding those of intact embryos at any stage of development. From the outset, therefore, there is more than enough respiratory apparatus to support any rate of respiration normally exhibited by a developing embryo, and the suggested explanation cannot be correct.

Another explanation, sponsored by Spiegelman and Steinbach, and also by Gregg and Ray, assumes that increasing respiratory norms are the direct result of processes progressively facilitating effective contact between respiratory enzymes and their substrates, *i.e.*, that the respiratory rate at any stage is limited simply by the rate at which some respiratory enzyme is able to combine with its substrate. But, under this assumption, we are left without good reason to expect an elevation of respiratory rate in the presence of uncoupling agents, and it is best abandoned.

Still another explanation (the one we shall adopt here) is provided by the theory outlined in the introduction. According to this theory, the respiratory norm is a function of the rate of energy expenditure; and waxing respiratory norms thus are to be ascribed to waxing rates of energy expenditure. In this connection it is known that developing *Rana pipiens* embryos are characterized by increasing rates of carbohydrate utilization (Gregg, 1948), and by increasing rates of turnover of labile phosphorus (Kutsky, 1950).

(2) Our mathematical analysis of the relation between developmental age and respiratory norm (*Rana pipiens*) has been made following the precedent established by Atlas (1938) and by Moog (1944), and the results of it are in considerable agreement with theirs. The following version of their equations is obtained by changing units to correspond with those used in the present investigation and by confining values of t to the interval (0–140):

(v) Atlas	$y = 3\,e^{0.038t}$	$(0 \leqslant t \leqslant 62)$
	$y = 16\,e^{0.019\,(t-62)}$	$(62 \leqslant t \leqslant 140)$
(vi) Moog	$y = 5\,e^{0.077t}$	$(0 \leqslant t \leqslant 50)$
	$y = 21\,e^{0.031\,(t-50)}$	$(50 \leqslant t \leqslant 140)$

Considering the nature of the supporting data, (i), (v) and (vi) are in good agreement. For *Rana pipiens* embryos, therefore, it appears to be established that respiratory norm is an exponential function of developmental age, and that respiratory acceleration decreases at some point in the age interval (50–62). For possible explanations of the acceleratory change, readers are referred to the papers of Atlas and Moog.

(3) We have already mentioned that *Rana pipiens* embryos are well-provisioned with respiratory substrates; therefore, on the basis of the theory outlined in the introduction, the stimulatory effect of DNP is to be expected.

(4) What is unexpected is the finding that the concentration of DNP eliciting the respiratory potential of *Rana pipiens* embryos is five times as great for the age interval (0–67) as for the age interval (72–140). There is no parallel for it in the development of sea urchin embryos, whose respiration is maximally stimulated at any stage by $5 \times 10^{-5}\,M$ DNP (Immers and Runnström, 1960); and, pending further investigation, it remains unexplained.

(5) There is no precedent to guide mathematical analysis of the data relating developmental age to respiratory potential (*Rana pipiens*); and subsequent investigation may necessitate revision of equations (iv), which have been obtained by taking the data at face value. For what it is worth, Figure 1 (upper curve) shows that the development of the respiratory potential of *Rana pipiens* embryos proceeds in three phases: two of exponentially increasing potential, separated by one whose characteristics are not known. The second phase may be one of constant potential,

as the data suggest; or, if the first and third phases are of greater duration than shown, it may be one of abruptly decreasing potential; or, although there is no reason for so believing, later work may show that the first and third phases actually intersect in the age interval (46–67), thus abolishing the second phase entirely. We shall leave the question open, after noting that Immers and Runnström (1960) observed a transient decline of respiratory potential in sea urchin embryos entering the mesenchyme blastula stage. But their result, also, was reported in a mood of skepticism.

In any case, it is clear that the respiratory potential of *Rana pipiens* embryos increases with age, remaining well above the respiratory norm, and thus maintaining a considerable margin of safety for energy expenditure. We have accounted for the developmental increase of respiratory norm by supposing that it is a function of increasing rate of energy expenditure. To account for increasing respiratory potential, it is necessary to assume the occurrence of intracellular structural changes progressively enhancing contact between respiratory enzymes and substrates. Data bearing upon this assumption are neither crucial nor consistent. Weber and Boell (1955) have found that the specific activity of mitochondrial cytochrome oxidase is an increasing function of developmental age (*Xenopus laevis*), thus indicating some process of mitochondrial differentiation; on the other hand, Spiegelman and Steinbach (1945) were unable to observe any developmental increase of the cytochrome oxidase activity of homogenates (*Rana pipiens*). Nevertheless, our assumption is supported by the electron microscopical study of Eakin and Lehmann (1957), who discovered profound developmental alterations of structural complexity and localization of the intracellular components, including mitochondria, of the ectoderm of neurulating amphibian embryos (*Xenopus laevis, Triton alpestris*). Therefore, until better assumptions are available, we shall adhere to our present one.

(6) The respiratory control quotient is a convenient measure of the degree to which the rate of energy expenditure holds the respiratory norm below the respiratory potential. Figure 1 shows that, during the first 46 hours of development, energy expenditure in *Rana pipiens* embryos is such as to permit a rapid approach of the respiratory norm to the respiratory potential; from 67 hours on, the relation is nearly stabilized, and respiratory norm is practically a constant fraction of respiratory potential. For reasons stated in the discussion of result (5), respiratory control quotients corresponding to the age interval (46–67) are left undefined. It is worth noting that a similar relation between respiratory norm and respiratory potential characterizes the development of sea urchin embryos (Immers and Runnström, 1960).

(7) We come now to the respiration of hybrid embryos. In a general way the data agree with those of Barth (1946) in showing that the respiratory norms of such embryos become increasingly subnormal as time goes on, and the same may be said of the rates at which they utilize carbohydrate reserves (Gregg, 1948). It appears, therefore, that they expend energy at increasingly subnormal rates; and this, on the theory of respiratory control we are adopting, is the reason for their progressively subnormal respiratory norms. On this basis, we should expect to find increasingly subnormal rates of turnover in their pools of labile phosphorus, but data are not yet available. There may be nothing wrong with their respiratory machinery, for their

homogenates respire at rates quantitatively similar to those of homogenates of *Rana pipiens* control embryos (Gregg and Ray, 1957).

(8) Our mathematical analysis of the relation between the developmental age and respiratory norm of hybrid embryos is based upon the assumption of linearity. The more precise data of Barth (1946) suggest that this assumption is not quite correct, but it is a useful approximation to the exact state of affairs. It should be noted that the intercept 6 of equation (iii) is in good agreement with the intercept 5 of equation (i).

(9) The view that the respiratory machinery of hybrid embryos may be entirely normal is supported by the finding that their respiratory rates are stimulated from 300 to 400% by 5×10^{-5} *M* DNP; for no better response is obtainable from *Rana pipiens* control embryos.

(10) This result need not be elaborated, except by pointing out that the concentration of DNP eliciting the respiratory potential of hybrid embryos at any stage is the same as that eliciting the respiratory potential of *Rana pipiens* pre-neurulae: there is no developmental shift in sensitivity to DNP like that exhibited by neurulating *Rana pipiens* embryos.

(11) On the assumption of linearity, the rate at which the respiratory potential of hybrid embryos increases is given by equation (iv). The increase of respiratory potential, though slower than normal, still is unnecessary; for the respiratory norms of hybrid embryos never overtake their initial respiratory potential (Fig. 2). Nevertheless, an increase of respiratory potential occurs; and to explain it we shall assume, in accordance with the discussion of result (5), that intracellular changes facilitating respiratory enzyme-substrate union take place in hybrid embryos as well as in normal ones, though at a much slower rate. Electron microscopical and biochemical studies of the intracellular particulates of hybrid embryos are much needed.

(12) This result does not require further commentary: reference to the discussion of result (6) will make its interpretation perfectly evident.

(13) This finding confirms the work of Gregg and Ray (1957): unless they are treated with a detergent (*e.g.*, deoxycholic acid), homogenates of very young *Rana pipiens* embryos do not respire at rates much different from the respiratory norm; but the respiratory rates of homogenates of progressively older embryos rapidly exceed the respiratory norm. We shall return to this topic in the discussion of the last result.

(14) The failure of DNP to elevate the respiratory activity of homogenates of *Rana pipiens* embryos at any stage of development is extremely puzzling, and no adequate explanation for it is now at hand.

Homogenates of adult tissues frequently do not exhibit a respiratory response to the presence of uncoupling agents. In such cases, respiratory rate appears to be limited, not by the rate of turnover of labile phosphorus, but by the low availability of readily oxidizable substrates; generally, this limitation is overcome by adding pyruvate, succinate or other respiratory metabolites (Krebs, 1959). This account of the matter is not applicable to homogenates of amphibian embryos (see the discussion of result (1)).

An arbitrary explanation, for which there is little independent support, may be constructed along the following lines. First, let us suppose that homogenization, by activating ATP-ase, results in some maximum elevation of the levels of ADP and

inorganic phosphorus. Second, let us suppose that homogenization is accompanied by the envelopment of respiratory particulates in lipo-protein or other envelopes. Third, let us suppose that the degree of envelopment decreases with developmental age. The first assumption guarantees a high rate of respiratory activity, other conditions permitting; and explains why DNP is without effect, the levels of ADP and inorganic phosphorus already being maximum. The second assumption explains why the respiratory activity of homogenates of young embryos is low, for we may expect that respiration under these conditions will be limited by the rate at which respiratory substrates are able to penetrate lipo-protein barriers. It also explains why the respiratory rate of homogenates of young embryos is elevated by detergents, for these may be expected to disperse lipo-protein deposits around respiratory particulates; or, even, to fragment those particulates (Siekevitz and Watson, 1956). The third assumption explains why the respiratory activity of homogenates is an increasing function of developmental age.

Summary

1. The respiratory rate of *Rana pipiens* control embryos is an increasing function of developmental stage, with an acceleratory change at the onset of the formation of the neural folds.

2. At any stage of development, the respiratory rate of *Rana pipiens* embryos is elevated by the presence of 2,4-dinitrophenol (DNP). The degree of stimulation obtainable ranges from about 400% of the control rate at the beginning of development to about 150% of the control rate at the gill-circulation stage.

3. The respiratory rate of *Rana pipiens* ♀ × *Rana sylvatica* ♂ embryos is an increasing function of time, but the rate of increase is very much lower than that of the respiratory rate of *Rana pipiens* controls.

4. At any stage, the respiratory rate of hybrid embryos is elevated by DNP. The degree of stimulation obtainable ranges from about 400% at the beginning of development to about 300% at 118 hours after fertilization (18° C.).

5. The respiratory activity of homogenates of *Rana pipiens* embryos at any stage is not altered by the addition of DNP.

6. The relevance of these findings to the question of embryonic respiratory control is discussed. It is concluded that, within the capacity to respire, respiration is governed by energy expenditure, and that the capacity to respire increases with age as the result of intracellular changes facilitating contact between respiratory enzymes and substrates.

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THE INFLUENCE OF SALINITY ON THE MAGNESIUM AND WATER FLUXES OF A CRAB

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The shore crab *Pachygrapsus crassipes* is known to be an osmotic regulator in both dilute and concentrated sea water, but its antennary glands are ineffective as organs of osmo-regulation inasmuch as the urine remains essentially isotonic to the blood, regardless of the salinity of the external medium (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a). On the other hand, Prosser *et al.* (1955) demonstrated Mg concentrations in the urine of this species when it is exposed to osmotic stresses, which suggests that the antennary glands are effective regulators of Mg. Gross (1959) confirmed these observations, but pointed out that the volume of urine produced, as well as the urine concentration of Mg, must be known before the antennary glands could be considered certain regulators of this cation. Prosser *et al.* (1955) also observed that while the urine concentration of Mg increased tremendously when the crab was immersed in increasingly saline media, the urine Na, contrary to expectation, decreased. They suggested that Na and Mg compete for transport across the membranes of the antennary gland with Mg predominating. When the crab was immersed in artificial Mg-free sea water equivalent to 170% of normal salinities, the observed concentration of Na in the urine was much higher than it was when the crab was immersed in 170% natural sea water, thus supporting the suggestion. However, the effects of such a treatment on the urine Mg concentration were not reported.

Even though *Pachygrapsus* is a strong regulator in large osmotic stresses, its blood tends toward concentrations which are intermediate between those it has in normal sea water and the concentration of the external medium (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a). Gross (1957a) demonstrated that such changes in the concentration of the blood are brought about by salts and not water; that is, the volume changes of the animal were insignificant. This must mean that either the formed tissues when bathed by such altered blood concentrations also remain unchanged in volume or that their volumes change at the expense of the blood space. Shaw (1955) demonstrated that the volume of muscle tissue in the hyper-regulating crab *Carcinus* increased when the animal was immersed in dilute sea water.

The present investigation will show that the efflux of Mg from *Pachygrapsus* is principally a function of water turnover and not due immediately to the Mg gradient between blood and external medium. It also will be shown that muscle tissue increases in volume when the crab is immersed in dilute sea water, and that it decreases in volume when the crab is immersed in concentrated sea water. This results in volume alterations in the blood space.

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Material and Methods

The lined shore crab, *Pachygrapsus crassipes* Randall, was collected at Dana Point, Laguna, and Ballona Creek, California. Only mature crabs of more than 15 grams were used. Care was taken that none was undergoing moult.

Blood and urine were sampled as previously described; analyses of Na and K were made by flame photometry and Ca and Mg by titration with ethylene diamine tetra acetic acid (EDTA) (Gross, 1959).

Artificial sea water was prepared according to the tables of Barnes (1954), normal sea water being considered to contain the following concentrations (meq./l.) of the major ions: Na, 460; K, 10; Ca, 20; Mg, 104; Cl, 538; SO_4, 56. The pH was adjusted to 8.0. In the Mg-free media Na was substituted for the deleted Mg and where Mg was increased above normal, relative to the other ions, Na was deleted accordingly to attain the desired osmotic pressure.

The effect of abnormal concentrations of medium Mg on blood and urine ionic concentrations was studied in two ways: (1) Groups of crabs from 100% natural sea water were immersed into small volumes of Mg-free artificial sea water of salinities equivalent to 50%, 100% and 150% of natural sea water, and into media equivalent to 50% in salinity but containing Mg equal to that of 100% natural sea water (104 meq./l.). Also, one group was immersed in a medium equivalent to 100% sea water in total salinity, but which contained half again as much Mg (156 meq./l.). All animals first were rinsed in the respective test medium before immersion. After 24 hours, the blood and urine of the experimental animals were analyzed for Na, K, Ca and Mg; the media which were originally Mg-free were analyzed for Mg. In the small volumes of media used for this group, the crabs could rise partially out of the water. This kept the mortality rate low over the 24-hour period of immersion, thus permitting a study of ionic alterations in the blood and urine in the artificial media.

(2) The second group of experiments was conducted primarily to measure rates of Mg excretion. Here animals were placed in large volumes of medium to assure complete and uniform immersion throughout the test period. Crabs previously acclimatized for 24 hours in natural sea water of salinities respective to their subsequent test media were immersed in large volumes of Mg-free 50%, 100% and 150% artificial sea water, these, again, having been rinsed first in the test media. During acclimatization in natural sea water, the crabs could rise out of the water. Again after a period of immersion ranging from one to six hours the blood and urine of the animals were analyzed for Na, K, Ca and Mg and the medium was analyzed for Mg. All experiments were conducted in a temperature-controlled room at 15° C.

In order to determine the volume changes in the muscle tissue of *Pachygrapsus* under different osmotic stresses, leg muscle from animals which had been immersed for three days in 50%, 100% and 150% natural sea water was rinsed in isotonic glucose and blotted uniformly. (Glucose concentrations were calculated from the tables in Gross, 1959.) These were then weighed and dried to constant weight in a drying oven at 95° C. The difference between dry weight and wet weight was considered to be the water content of the tissue.

Changes in the blood space volume were shown by calculating the volume of distribution of C^{14}-tagged sucrose in the blood space one minute after injection. Crabs removed from immersion for three days in 50%, 100% and 150% natural sea

water were injected at the base of the fourth walking leg with 0.3 to 0.5 ml. (depending on the size of the crab) of 1 *M* sucrose which had been tagged with C^{14}.

About one minute after the injection, a sample of blood was taken from the opposite side of the animal and diluted with 5 ml. of 1 *M* untagged sucrose. The quantity of blood was determined by weight and averaged about 0.3 gram. Then a 0.10-ml. aliquot of the diluted blood was absorbed onto a filter paper disc, allowed to dry and counted on a Nuclear-Chicago scaling unit. Mean counts thus obtained for three discs made from each diluted blood sample were compared with the mean counts of three 0.10-ml. aliquots of the dose which were plated in the same manner. Since the blood was diluted in 1 *M* sucrose and the dose was 1 *M* sucrose, the error

TABLE I

Ion concentration in the urine of Pachygrapsus *immersed in small volumes of artificial sea water*

		50%			100%			150%		
		Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.
Na (meq./l.)	N	380	60	37	378	64	15	353	106	30
	0	400	28	12	332	96	12	430	81	15
	+	401	33	25	319	80	8			
K (meq./l.)	N	10.0	3.5	37	7.8	1.4	15	9.6	1.1	30
	0	14.3	8.7	12	15.9	8.0	12	16.0	4.2	15
	+	11.0	3.8	25	7.0	2.0	8			
Ca (meq./l.)	N	32.7	7.1	31	36.0	6.3	15	47.9	5.2	20
	0	34.5	4.5	13	34.5	4.5	13	47.6	18.6	15
	+	31.1	8.7	19	37.0	7.8	14			
Mg (meq./l.)	N	70.5	41	31	305	130	15	408	122	29
	0	85.0	42	16	298	104	13	281	85	15
	+	65.5	23	18	368	101	14			

N = crabs immersed in concentrations of natural sea water.
0 = crabs immersed in Mg-free sea water.
+ = crabs immersed in artificial sea water containing abnormally high Mg: in 50% sea water = 104 meq./l.; in 100% sea water = 156 meq./l.

due to self-absorption should be essentially the same in radio-assays of both blood and dose. Care was taken to assure uniform geometry. At least 1000 counts were observed for each sample, and maximum rates did not allow significant coincidence.

The volume of distribution for sucrose in one minute was, therefore, calculated from the equation: $V = \frac{d/b}{w} \times 100$

where V = volume of distribution of sucrose in one minute (% body weight);
d = observed total activity (counts/min.) injected into the crab;
b = observed activity (counts/min.) per gram of blood;
w = weight of crab (g.).

Also, a correction was applied for the volume of the dose.

After the blood samples were taken, the crabs were placed in a closed chamber

containing a $Ba(OH)_2$ trap designed to absorb CO_2. Radio-assay of the total precipitate thus collected in 24 hours for ten crabs demonstrated no significant activity, suggesting that the sucrose was not metabolized. It is possible that some was fixed in the body, but it seems more likely that it remained in solution in an unchanged state at least for the brief period (one minute) during which the dilution was being observed.

Results

Table I compares the urine Mg of crabs immersed in small volumes of artificial sea water with the urine Mg of crabs immersed for 24 hours in small volumes of natural sea water. The value for crabs in 100% natural sea water is higher than previously reported, but is believed to be more reliable because of improved technique in sampling the urine; the values for crabs in 50% and 150% natural sea water have been reported previously (Gross, 1959). It is clear from these data that when *Pachygrapsus* is immersed in 50% and 100% Mg-free artificial sea water, the concentration of Mg in its urine is close to the concentration of Mg in the urine of crabs immersed in the same salinities of natural sea water. When a crab is immersed in 50% sea water for 24 hours, the Mg concentration of its urine is about the same whether the medium Mg is 0, 52 or 104 meq./l. In salinities of 100% normal, the observed mean concentration for urine Mg was about the same for animals immersed in Mg-free water as for those from natural sea water; for animals immersed in 100% artificial sea water containing abnormally high Mg, the urine Mg was slightly higher than for crabs from 100% natural sea water (368 meq./l. to 314 meq./l., respectively), but these means are not significantly different (Table I). The urine Mg of crabs immersed in small volumes of Mg-free 150% sea water was less than that for crabs from 100% or 150% natural sea water. This is difficult to interpret, but possibly could be explained as a reflection of the crab's greater tendency to remain out of the artificial medium. Such an argument is supported by the fact that the urine Mg for crabs immersed in large volumes of Mg-free 150% sea water averaged about the same (470 meq./l.) as did crabs from small volumes of 150% natural sea water (408 meq./l.). Also, when the crabs were immersed in large volumes of 100% Mg-free sea water, the urine Mg was about the same as for crabs immersed

Table II

Excretion of Mg by Pachygrapsus *in stress media*

Test medium	Medium volume*	Mean urine Mg meq./l.	S.D.	No.	Mean Mg excreted meq./day/g.	S.D.	No.
150% sea water without Mg	small (24 hrs.)	281	85	15	0.0021	0.001	14
	large (6 hrs.)	471	152	17	0.0073	0.0054	18
100% sea water without Mg	small (24 hrs.)	298	104	13	0.0070	.0033	14
	large (6 hrs.)	243	103	15	0.0095	0.0084	15
50% sea water without Mg	small (24 hrs.)	85.0	42	16	0.0066	0.0030	20
	large (1 hr.)	72.0	44	12	0.0433	0.022	12

* Time in parentheses = period of immersion.

in 100% natural sea water (Tables I and II). Table III presents probability values for analyses of Mg excretion.

It might be argued that the animals immersed in the larger volumes of artificial sea water were previously acclimatized to the respective salinities, and that sufficient time had not been given to permit alteration of the urine concentrations. However, when 13 crabs were transferred directly from 100% natural sea water and immersed for six hours in a large volume of 150% Mg-free sea water, the mean urine Mg was 713 meq./l., indicating not only that considerable changes can occur in the urine Mg concentration in six hours, but also that the greater hyperosmotic stress due to transferring the animals directly from 100% natural sea water to 150% artificial sea water probably made the urine even more concentrated with respect to Mg, again independently of the influx of this ion from the medium. Thus, for the periods

TABLE III

Probability values for analyses of Mg excretion

A. Comparison of urine Mg concentrations: crabs immersed in artificial sea water vs. crabs immersed in natural sea water

	50% Sea water		100% Sea water		150% Sea water	
	Large volume	Small volume	Large volume	Small volume	Large volume	Small volume
Mg-free	>0.50	>0.20	=0.20	>0.50	>0.10	<0.001
Excess Mg		>0.50		>0.20		

B. Comparison of rates of Mg loss by crabs completely immersed in Mg-free artificial sea water

100% S.W. vs. 150% S.W.	>0.30
100% S.W. vs. 50% S.W.	<0.001
150% S.W. vs. 50% S.W.	<0.001

indicated the concentration of Mg in the urine is not determined by the influx of this ion from medium, but rather, at least indirectly, by the osmotic pressure of the external medium.

Table I also compares the urine concentrations of the other three major cations of the crabs immersed in small volumes of artificial sea water with those immersed in the respective salinities of natural sea water. Contrary to the findings of Prosser *et al.* (1955), there is no dramatic increase in urine Na when Mg is deleted from the medium. Although in 100% artificial sea water with high Mg the urine Na of *Pachygrapsus* was somewhat low, this could be accounted for by the low Na in the medium rather than the high Mg. Then in 150% Mg-free sea water the urine Na was high, but again this was likely due to the high Na in the medium, substituting for the deleted Mg. The concentration of Ca in the urine also seems unaffected by the absence or relative increase of Mg. With regard to K, the urine concentrations of this ion are significantly higher when the animal is immersed in 100% and 150% Mg-free sea water, than when immersed in the same concentrations of natural sea water ($P < 0.01$). The mean urine K for animals immersed in 50% Mg-free sea water also was higher than for crabs immersed in 50% natural sea water, but the difference cannot be shown to be significant. Neither is the urine K of animals

immersed in 50% sea water with normal Mg concentrations (104 meq./l.) significantly different from that of crabs from 50% natural sea water.

Table II reveals the rate of Mg excretion in the different Mg-free salinities. Thus, considering only those crabs immersed in the large volume where they could not rise out of the water, it can be seen that the mean Mg excreted in 100% sea water is greater than the mean Mg excretion in 150% sea water, but the difference between these means is not significant (Table III). On the other hand the rate of Mg loss is four times as great in 50% sea water as it is in normal sea water; this difference is highly significant, $P < 0.001$ (Table III). Table II also shows that less Mg is lost to a small medium than to a large one. This, of course, would be expected because the animals could rise out of the small volume. Among all test media the difference in rates of Mg excretion between large and small volume treatments was smallest for 100% sea water, suggesting that in this salinity there is a minimum attempt to rise out of the water. The tendency for this crab to avoid an osmotic stress has been noted previously (Gross, 1957b).

The concentration of Mg in the urine of crabs immersed in large volumes of artificial sea water also is given in Table II. It will be recalled that these crabs first were acclimatized to the respective salinities of natural sea water before treatment in artificial sea water. Thus, there was no large change in the osmotic gradient to which the animal was subjected following acclimatization. The indicated periods of immersion were chosen because after such time in the test media, the urine Mg did not differ significantly from that of crabs removed from the respective acclimatizing salinities of natural sea water. Immersion periods of more than one hour in large volumes of Mg-free 50% sea water apparently deplete the Mg supply of the crab and the urine Mg becomes greatly reduced in concentration. Mg excretion of *Pachygrapsus* immersed for one hour in all three salinities of Mg-free sea water could not be compared because in this brief time insufficient amounts of the ion were released in 100% or 150% sea water to be detected with precision by the methods available.

If, then, the consistent concentration of Mg in the urine were known throughout a period of immersion and the amount of Mg lost to the medium in that period also were known, then assuming that the antennary glands are the sole pathways of Mg efflux, the volume of urine necessary to excrete the observed loss of Mg can be calculated. Estimations of urine flow from mean rates of Mg loss and mean Mg concentrations in the urine follow: in 150% sea water the volume of urine production was calculated to be 1.5% body weight/day; in 100% sea water, 3.9% body weight/day and in 50% sea water, 58% body weight/day.

The value for *Pachygrapsus* immersed in 100% sea water compares favorably with values reported by Webb (1940) on *Carcinus* and by Robertson (1939) for *Cancer* when the crabs were immersed in normal sea water, but only half the value reported by Nagel (1934) for *Carcinus* immersed in normal sea water. These workers, however, plugged the nephropores and assumed the gain in weight was due to urine which could not escape. Values obtained by three different methods on the prawn, *Palaemonetes,* in 100% sea water were more than twice as large as the above rate for *Pachygrapsus* (Parry, 1955).

As might be expected, the calculated rate of urine flow for *Pachygrapsus* was less in 150% sea water than in 100% sea water and the rate in 50% sea water

greater than in normal sea water, but of such magnitude (58% body weight/day) that it is subject to question. This rate was determined on the basis of Mg excretion after one hour total immersion, but since the crabs had been acclimatized to 50% natural sea water before treatment in the Mg-free medium, there was no large increase in the osmotic gradient between external medium and the blood of the animal. However, forced total immersion, which did not take place in the acclimatizing procedure, caused more surface of the crab to be exposed to stress and this probably caused an increase in the water influx and the consequent increase in urine flow. It should be emphasized again that the concentration of Mg in the urine following immersion in large volumes of 50% Mg-free sea water averaged about the same as for crabs immersed for 24 hours in 50% natural sea water. It is thus likely that the urine Mg concentration for this group of crabs did not change during the one-hour immersion period. Either, then, the rate of urine flow for crabs thus treated is as calculated (58% body weight/day) during that period of immersion, or in such a hypotonic medium, mechanisms of Mg loss are different from those in crabs immersed in 100% and 150% Mg-free sea water. The Mg gradient between blood and external medium (Mg-free) would be about the same for all three conditions; yet Table II demonstrates the greatest mean loss to 50% and the smallest mean loss to 150% sea water. It is our opinion that if the principal pathway for Mg loss in all the above conditions were the antennary glands, then the above value for the rate of urine flow is a fair approximation for the conditions described. It would follow that such a rate could not be sustained, and it is interesting that the number of fatalities for crabs immersed for one hour in the large volume of 50% sea water was twice as large as the combined number of fatalities for crabs totally immersed for six hours in 100% and 150% sea water. Also, urine Mg in a few crabs which survived for six hours in 50% Mg-free sea water was essentially nil, and the actual amount of Mg lost to the medium was about twice that lost by crabs immersed in large volumes of Mg-free 100% sea water for the same period. This indicates exhaustion of the Mg supply in the crabs. When nine crabs which had been acclimated for 24 hours in small volumes of 50% natural sea water were transferred to large volumes of the same medium, for six hours, the mean urine Mg dropped to 47.2 meq./l., S.D. = 13.4. This is significantly less than the urine Mg of crabs from small volumes of 50% natural sea water (Table I): $P = 0.01$. It is suggested that sudden total immersion in natural 50% sea water causes a depletion of Mg reserves more rapidly than they can be replenished from a medium of this salinity. The low mortality rate in this group of crabs also suggests that Mg depletion is a cause of death in the crabs immersed in Mg-free 50% sea water, but also raises the question as to how long *Pachygrapsus* can survive totally immersed in 50% sea water in nature.

In order to estimate the urine flow when *Pachygrapsus* was removed from water, ten crabs were taken from normal sea water, rinsed in distilled water to wash away residual salts, then blotted dry. The crabs then were placed in dry containers and kept in a relatively humid temperature-controlled room at 15° C. for 72 hours. Then the crabs and their containers were rinsed with distilled water and the washings saved for Mg analysis. Also, the urine from these animals was sampled and analyzed for Mg. The urine concentration for Mg and the total excretion of Mg into the container then should yield the volume of urine flow

during the 72-hour period. The average calculated rate of urine produced, thus determined, was 0.02% body weight/day, which is hardly significant.

Table IV demonstrates the effects of altered Mg in the medium on the blood concentration of the four major cations in *Pachygrapsus*. As would be expected, the blood Mg concentrations of crabs immersed in 100% and 150% Mg-free sea water are less than those of crabs from the same salinities of natural sea water. However, the blood Mg of crabs immersed in Mg-free 50% sea water was not

TABLE IV

Ion concentrations in the blood of Pachygrapsus *immersed in small volumes of artificial sea water*

		50%			100%			150%		
		Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.	No.
Na (meq./l.)	N	397	24	37	483	17.3	36	582	34	30
	0	410	30	15	493	9.6	14	562	40	15
	+	410	30	21	478	15.1	8			
	N/0	0.97			0.98			1.04		
	N/+	0.97			1.01					
K (meq./l.)	N	7.36	1.4	37	7.43	0.72	36	10.2	1.5	30
	0	5.78	1.0	15	9.19	0.82	14	8.62	1.4	15
	+	6.53	1.0	21	7.88	1.4	8			
	N/0	1.27*			0.81*			1.19*		
	N/+	1.13			0.94					
Ca (meq./l.)	N	34.8	7.9	24	29.6	5.9	44	36.4	4.8	30
	0	33.1	9.6	14	32.0	8.1	14	40.5	6.3	15
	+	29.7	6.4	16	38.1	8.4	17			
	N/0	1.05			0.93			0.90		
	N/+	1.17			0.78*					
Mg (meq./l.)	N	13.6	5.4	24	20.0	6.1	44	27.1	4.2	30
	0	13.2	5.1	14	11.7	5.7	14	16.7	7.5	15
	+	19.7	7.4	16	33.2	6.1	15			
	N/0	1.03			1.71*			1.62*		
	N/+	0.69*			0.60*					

N = crabs immersed in natural sea water.

0 = crabs immersed in Mg-free artificial sea water.

+ = crabs immersed in artificial sea water containing abnormally high Mg: in 50% sea water = 104 meq./l.; in 100% sea water = 156 meq./l.

* = significantly different from unity: $P < 0.01$.

significantly less than the blood Mg for crabs from 50% natural sea water. On the other hand, blood Mg for crabs immersed in 50% artificial sea water which contained normal Mg (104 meq./l.) was about equal to the blood Mg of crabs from 100% natural sea water (20 meq./l.). Also, the mean blood Mg of crabs immersed in 100% artificial sea water with high Mg (156 meq./l.) was 33.2 meq./l., which is significantly higher ($P < 0.01$) than the concentration of this ion for crabs which had been immersed in either 100% or 150% of natural sea water (20.0 and 27.1 meq./l., respectively). This is particularly interesting because as

indicated above, the urine Mg of crabs from this artificial medium is about equal in concentration to that of crabs from 100% natural sea water. It is apparent that the blood concentration of Mg is influenced by the influx of Mg from the medium, even though the Mg concentration in the urine is not directly affected.

Table IV also shows that blood Na is essentially unaltered by abnormal concentrations of Mg in the medium. It is interesting, however, that when immersed in 100% artificial sea water containing high Mg, the blood Na of *Pachygrapsus* remains normal, even though the concentration of this ion in the medium was reduced because of the high Mg. Blood Ca is unaltered in all conditions except when the crab is immersed in 100% sea water with high Mg. Here the blood Ca is significantly higher ($P < 0.01$) than for crabs from 100% natural sea water. There seems to be an interdependence between the regulation of Ca and Mg under these conditions. In Mg-free 50%, 100% and 150% sea water the blood K differs from the blood K of crabs from natural sea water. There is no consistent trend,

TABLE V

Apparent volume of distribution of sucrose in the blood space and water content for muscle in* Pachygrapsus *following immersion in different salinities*

Test medium (% sea water)	Mean volume of distribution (% body wt.)	S.D.	No.	Mean water content of muscle (% wet wt.)	S.D.	No.
50	15.4	1.39	10	76.58	1.77	18
100	18.7	3.45	11	75.00	1.40	25
150	26.7	5.34	14	71.70	1.71	34

* One minute after injection.

but it can be suggested that there also is an interdependence between the regulation of Mg and K. Such a suggestion is supported by the above mentioned differences in urine K between animals from Mg-free sea water and those from natural sea water.

Table V demonstrates that the muscle tissue of *Pachygrapsus* gains water when the animal is immersed in 50% sea water and loses water when it is immersed in 150% sea water. That is, the muscle is permeable to water in both directions. This is particularly interesting inasmuch as the animal itself shows no significant weight changes during such treatments (Gross, 1957a). Table V also shows that the calculated apparent volume of distribution for sucrose one minute after injection into the blood space is smallest when the crab is in 50% sea water and largest when the crab is immersed in 150% sea water. This is interpreted to mean that the blood space volume of *Pachygrapsus* is altered when the animal is transferred from one salinity to another by the changing volume of the formed tissues. There may be objections to the use of only one concentration of sucrose for the injected dose (1 *M*), but the volume of the dose was no greater than 0.5 ml. and, if anything, would be expected to cause an increase in blood volume for crabs from dilute sea water and a reduced blood volume for crabs from concentrated sea water.

Discussion

The diagram presented in Figure 1 suggests the course of events with respect to Mg and water fluxes when *Pachygrapsus* is immersed in different concentrations of sea water. Since the indicated Mg values are based on the crab's response to Mg-free media, it can be seen that the concentration of Mg in the urine of

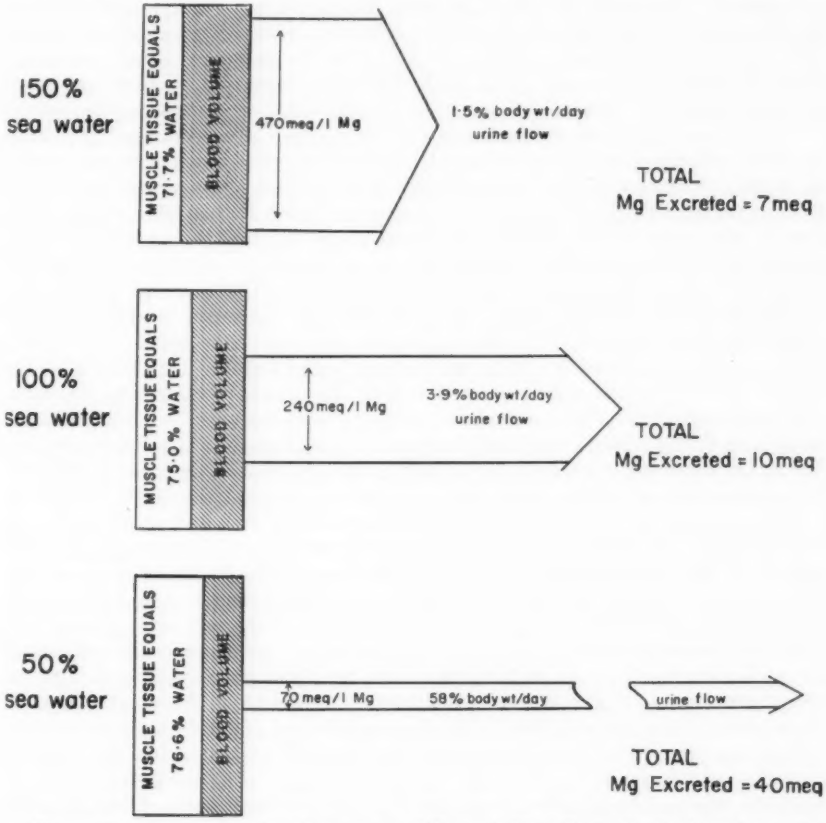

Figure 1. Scheme of urine flow, water shifts and Mg effluxes in *Pachygrapsus* when exposed to different osmotic situations (indicated by the concentration of the external medium in % sea water on left side of diagram). Arrows which represent urine flow and Mg efflux are based on mean values for Mg concentrations in the urine and Mg losses to the medium when the crab is completely immersed in 50%, 100% and 150% Mg-free artificial sea water (see Table II). Length of arrow = volume of urine flow (% body weight/day); width = urine concentration of Mg (meq./l.); arrow area = relative amount of Mg excreted (meq.). Rectangles which are not drawn to scale represent crabs and illustrate the differences in blood volume (hatched area) in different osmotic situations, as suggested by the calculated volume of distribution for sucrose (Table V), and the volume of formed tissue (blank area), as suggested by the water content of muscle for crabs from different osmotic situations (Table V).

Pachygrapsus is directly related to the salinity of the external medium and not to the concentration of Mg in the medium or its influx into the animal. Likewise, it is shown that the efflux of Mg is inversely related to the concentration of this ion in the urine. (While the mean Mg loss in 150% was less than the mean Mg loss in 100% sea water, these values are not significantly different. However, the mean Mg loss in 50% sea water was more than four times the loss in the other two media.) Assuming that the antennary glands are the principal pathways for Mg efflux for all conditions, then small volumes of urine are produced in concentrated sea water and large volumes of urine are produced in dilute sea water. Figure 1 likewise shows that when *Pachygrapsus* is immersed in dilute sea water the water content of formed tissues is higher than when it is immersed in normal sea water, and conversely when the crab is immersed in concentrated sea water the water content of its formed tissues is less than when it is immersed in normal sea water. Such changes in water content effect volume alterations in the tissues at the expense of the blood space. As shown in Figure 1, then, the concentration of urine Mg is apparently determined by the water flux and is immediately independent of the concentration of this ion in the medium. Also, it is relatively independent of the Mg concentration in the blood. It will be observed (Table IV) that the blood Mg of crabs immersed in Mg-free sea water can be reduced below normal, yet the urine Mg (Table I) remains high (*e.g.* in 100% sea water). Again, in crabs immersed in 100% artificial sea water with high Mg the blood Mg becomes elevated above normal, but the urine Mg remains essentially the same as it is in crabs from 100% natural sea water. This response is contrary to the findings of Webb (1940), who demonstrated in *Carcinus* in a similar experiment that increases in the concentration of Mg in the external medium were reflected in the urine, but very little in the blood. Thus the mechanism of Mg regulation in *Pachygrapsus* may be fundamentally different from that of *Carcinus*. In the latter case the concentration of Mg in the urine increased even though the total salinity of the medium was normal; yet the blood Mg remained close to the concentration it attains in crabs from 100% natural sea water. As proposed by Webb (1940), excretion of Mg in *Carcinus* by way of the antennary glands does depend on the influx of this ion from the medium and consequently on the concentration of Mg in the blood. On the other hand, in *Pachygrapsus*, as shown above, the concentration of Mg in the urine is relatively independent of both the influx of this ion from the external medium and the concentration of Mg in the blood *per se*.

It seems, then, that in *Pachygrapsus* the concentration of urine Mg depends inversely upon the rate at which the antennary glands form urine or the rate at which the Mg-containing fluid is transported across the membranes of the antennary glands. This, in turn, depends on the magnitude and direction of water flux between the animal and its external medium.

The volume of urine flow calculated from the rates of Mg excretion seems reasonable for crabs immersed in 100% sea water. At least this value (3.9% body weight/day) agrees favorably with other values in the literature on other species, suggesting that this may be a valid method for estimating urine production. It is interesting that in a crab completely immersed in 150% sea water the calculated urine flow is 1.5% body weight/day, because there is a tendency for the crab to lose water to the hypertonic medium, yet there is no significant weight change

even though fluid is being lost by way of the urine and probably by diffusion. The animal, therefore, must possess a mechanism for actively taking up water. Drinking is suggested as the principal method for replacing lost fluid. Burger (1957) has demonstrated drinking in *Homarus;* Green *et al.* (1959) have produced evidence that the gut takes part in the hypo-osmotic regulatory mechanism of *Uca.*

The excessive calculated urine flow for crabs completely immersed in 50% sea water (58% body weight/day) is difficult to interpret. It does not seem that such a rate could be sustained for long. This must mean that should this species inhabit water of such low salinities for prolonged periods, it must either alter its permeability or perhaps, being free to come out of the water, limit its period of immersion. The calculated rate of urine flow in the small volume of 50% sea water where the crab could rise out of the water was less than 10% body weight/day.

Again it becomes clear that the concentration alone of a given ion in the urine does not reveal the relative rate of excretion of that ion or, in the case of crabs, the relative quantitative role of the antennary glands in regulation of a particular substance. Table II reveals that the greatest mean loss of Mg was by crabs immersed in 50% sea water; yet these same crabs possessed the lowest concentrations of Mg in the urine. Conversely, the smallest mean loss of Mg was by the crabs immersed in 150% sea water which in turn had the highest Mg concentrations in the urine (Table II, Figure 1). In the case of crabs kept out of the water, the concentration of urine Mg was high (300 meq./l.), yet the amount excreted in three days could hardly be measured.

It is suggested from the lack of Mg loss when the crab is out of the water that *Pachygrapsus* must depend upon an uptake of water in order to excrete urine. This, then, is another physiological limitation binding this semi-terrestrial species to the sea. Gross (1955) discusses other characters which limit the terrestrial life of this crab.

It has long been recognized that when an aquatic animal enters a medium of a different salinity, it must undergo certain physiological adjustments which include the mechanisms of tolerance permitting adequate functioning of the cells and tissues despite changes in the salt concentration of the surrounding body fluids, or regulation which keeps those changes in blood concentrations at a minimum. Evidence has been produced by the present investigation that *Pachygrapsus* is capable of regulating the total water content of its body by expelling the excessive influx when in a hypotonic medium by a rapid flow of urine and, conversely, compensating in some way for the physical efflux of water when immersed in a hypertonic medium (perhaps by drinking). Table V, however, shows that the muscle tissue cannot regulate its volume, at least not during a three-day exposure to osmotic stress, the result being that the anatomy of the vascular system also becomes altered. It does not seem that the changes in the volume of the blood space suggested by data in Table V would not affect the efficiency of the animal.

It would seem, therefore, that should *Pachygrapsus* inhabit salinities which vary much from those of normal sea water for prolonged periods, it would be obliged to control the volume of its formed tissues so that a normal and efficient anatomy could be assured for the vascular system.

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We wish to thank Mr. John Bristow and Miss Mary Conlee for their able technical assistance. Also, we wish to express our gratitude to all those students who assisted in collecting the experimental animals.

Summary

1. The concentration of Mg in the urine of *Pachygrapsus* is dictated by the salinity of the external medium and not by the Mg concentration in that medium or by the rate of Mg influx from the medium into the animal. Thus, during brief periods of immersion in 50%, 100% or 150% sea water the urine Mg concentration will reflect the salinity of the medium, irrespective of whether Mg is absent or in abnormally high concentrations.

2. The Na concentration in both blood and urine is not drastically altered by abnormal Mg levels in the external medium of any salinity tested.

3. After immersion in Mg-free 100% and 150% sea water the urine K of *Pachygrapsus* is higher than it is after immersion in the respective concentrations of natural sea water. Urine K is not influenced by the Mg concentration of 50% artificial sea water or by abnormally high Mg in 100% sea water. Blood K concentrations are affected by varying concentrations of Mg in the external medium of both dilute and concentrated salinities, but there is no definite trend.

4. The concentration of Ca in the urine of *Pachygrapsus* is unaffected by the Mg levels of all salinities tested. Blood Ca was not observed to be altered by abnormally high or low Mg levels in all media tested except in 100% artificial sea water with high Mg (156 meq./l.), where the blood Ca was significantly higher than for animals from 100% natural sea water.

5. While the concentration of urine Mg is not determined immediately by the influx of this ion into the animal, the blood Mg concentration is lowered when the crab is immersed in a Mg-free medium and raised when the medium Mg is abnormally high. The concentration of urine Mg is relatively independent of the levels of Mg in the blood.

6. *Pachygrapsus* excretes more Mg in 50% sea water than in 100% sea water and perhaps less in 150% sea water than in 100% sea water, even though the concentration of Mg in the urine is in the reverse order (*i.e.*, 150% > 100% > 50%).

7. Calculated rates of urine production for *Pachygrapsus* completely immersed in different salinities follow: in 150% sea water, 1.5% body weight/day; in 100% sea water, 3.9% body weight/day; in 50% sea water, 58% body weight/day. The observed rate for crabs immersed in 50% sea water is not believed to be sustained for long, as suggested by the high mortality rate. When removed from water, the volume of urine excreted by *Pachygrapsus* is insignificant.

8. The concentration of urine Mg in *Pachygrapsus* thus is inversely related to the rate at which urine is produced by the antennary glands and this is dependent on the magnitude and direction of the water flux, imposed by the physical gradient between the crab and its external medium.

9. The volume for muscle tissue in *Pachygrapsus* increases when the crab is transferred from normal sea water to dilute sea water and decreases when it is transferred to concentrated sea water. Such volume changes take place at the expense of the blood space.

10. It is suggested that alterations in the volume of the blood space caused by osmotic stress likely would reduce the efficiency of the vascular system which in turn would impose further ecological limitations on this species.

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NEUROMUSCULAR PHYSIOLOGY OF A SESSILE SCYPHOZOAN

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Interest in the coelenterate neuromuscular system has persisted for many years, and information gained by a variety of approaches. Pantin (1935a, 1935b, 1935c, 1935d) first introduced the use of controlled electrical stimuli and the observation of subsequent muscular contractions as a means of analysis of the properties of the conducting system in Actiniaria. Bullock (1943) has shown that the propagation of the swimming pulse in Scyphozoa differs in no fundamental way from excitation in anemones, although there are certain quantitative differences that result in the remarkable dissimilarity of behavior of the two groups of organisms. In anemones, contraction of the muscles is relatively rapid, but relaxation is slow, and because of neuromuscular facilitation each impulse after the first arriving at the muscle excites further contraction so that a typical "staircase" is recorded from the contraction of the sphincter muscle in *Calliactis*. In scyphozoan medusae the first impulse is effective in eliciting a response. Each succeeding impulse, if delivered within a well defined time limit, enhances the response for several contractions until a plateau is reached which may be maintained for long periods of time. After each contraction the muscle relaxes completely (or almost so) so that the typical kymograph record appears as in Figure 1, a recording of *Cyanea* bell contractions. This is the result of rapid contraction, rapid relaxation, the restoring force of the mesoglea, and an extraordinarily long absolute refractory period (0.7 second, according to Bullock, 1943) of the muscles of the medusa bell.

As indicated above, all critical work using controlled electrical stimulation has been confined to Anthozoa and free-swimming jellyfish. This raises the question of the relationship of response to mode of life. Are the phenomena described sharply divided along systematic lines, or are there some definite and demonstrable properties that may be associated with the sessile habit or the free-swimming habit? The Stauromedusae, about which very little is known, offer ideal material for such a study.

Except for a brief creeping larval phase, Stauromedusae are sessile throughout their existence, but there is no doubt of their affinities with the Scyphozoa. Do they retain the neuromuscular patterns of their nearest relatives, or has the sessile habit led to modifications (or retentions from the scyphistoma larva) that make the Stauromedusae functionally more closely associated with the anemones? Although Stauromedusae lack the ability to perform pulsating swimming motions, one might wonder if the system is capable of such contractions under artificial stimulation. Is the absence of a pacemaker the only essential factor here?

The comparative aspect of the problem would be ideally developed by studying:

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(a) the responses of anemones; (b) the responses of free-swimming Scyphozoa; (c) the responses of the scyphistoma larva of the Scyphozoa; and (d) the responses of Stauromedusae. A knowledge of all of these might enable one to assess the consequences of the sessile habit and give some insight into the evolution of their neuromuscular mechanisms and behavior. The present study deals only with the last of these.

Materials and Methods

Haliclystus auricula (Rathke) was used because of its abundance near the Friday Harbor Laboratories of the University of Washington. Large individuals were collected, along with a portion of the blade of *Zostera* to which they are normally attached. The animals were maintained in the laboratory in running

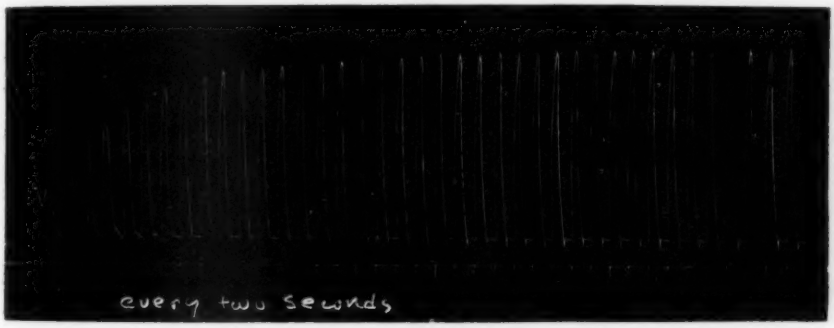

Figure 1. *Cyanea capillata.* Record of bell contractions. Electrical stimuli, 0.5 per second.

sea water at a temperature close to that of their natural environment (10–13° C.). Reactions were first explored by means of manually controlled mechanical stimuli and later by controlled condenser discharge shocks. Recordings were made with very light isotonic levers on a smoked drum.

Electrical stimuli were delivered under sea water to the exumbrellar surface, usually at the base of the stalk or at the stalk-calyx junction, by means of silver electrodes insulated to the tip, with Ag-AgCl-sea water electrodes or non-polarizable calomel electrodes. A student "Electrodyne" stimulator, which delivers brief condenser discharge shocks, and for some experiments a Grass model S4B stimulator, were used. All experiments were made on fresh animals at temperatures of 11–13° C. At temperatures above 16–18° C. responses became very erratic, probably due to the rapid decay of facilitation (Hall and Pantin, 1937; Pantin and Vianna Dias, 1952). Mechanical stimuli were delivered with a clean, blunted glass rod or with a glass rod tipped with a short length of silver wire.

Anatomical studies to determine the details of the musculature were carried out by observation on entire living and preserved animals under reflected and transmitted light and between crossed polaroids. To confirm the results obtained from a study of entire animals, portions of several were sectioned and stained

with picro-indigo-carmine following bulk staining in Grenacher's borax carmine, or with alum hematoxylin and eosin.

I would like to express my gratitude to the Director and Staff of the Friday Harbor Laboratories of the University of Washington for a grant which made residence at the Friday Harbor Laboratories possible for a period of seven months. My thanks are also due to the Department of Zoology, University of California, Berkeley, for support and encouragement over a period of several years. Dr. Ralph I. Smith and Dr. Cadet Hand have aided in many important ways, not the least of which was a critical reading of the manuscript. Their interest and criticism is deeply appreciated. Dr. T. H. Bullock has been kind enough to read the manuscript. His criticisms and suggestions have been very helpful and are gratefully acknowledged.

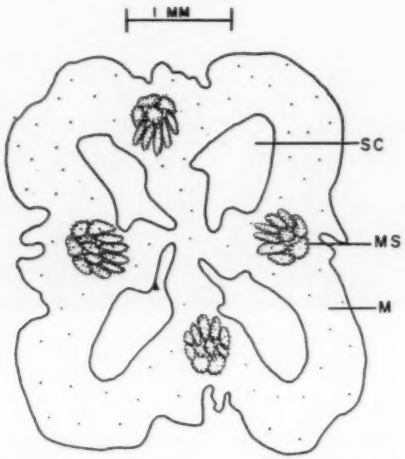

FIGURE 2. Cross-section through the stalk of *Haliclystus auricula*. SC, stalk canal; MS, stalk muscle; M, mesoglea.

RESULTS

Musculature of Haliclystus

It is possible to divide the muscles of *Haliclystus* into three categories associated with particular regions of the body. The stalk possesses four interradial muscular bundles (Fig. 2) embedded in the mesoglea which continue distally into the subumbrellar ectoderm as eight perradial sheets that fan out over the subumbrellar surface (Fig. 3). Proximally, the stalk muscles are inserted at the pedal disc over a rather wide area. There is also a marginal muscle (subumbrellar) which borders the margin of the calyx (Fig. 3) and is only partially embedded in the mesoglea. This latter muscle is interrupted at the arms and so consists of four perradial and four interradial segments. In addition, arm tentacles, gastric tentacles, manubrium, and anchors are provided with muscle fibers. The principal

muscles involved in the reactions studied, however, are those of the stalk, subumbrella, and margin. With the possible exception of the stalk musculature, all the muscles of *Haliclystus* are confined to the subumbrellar surface, just as in free-swimming Scyphozoa. Thiel (1936) states that there are probably fine muscle fibrils distributed all over the body, although their existence has not been established, and no particular function has been ascribed to them. I have been unable to determine the presence of exumbrellar muscle fibers in *Haliclystus* and believe that all of the gross actions of the organism can be explained on the basis of interactions of the well-defined muscle fields and the mesoglea.

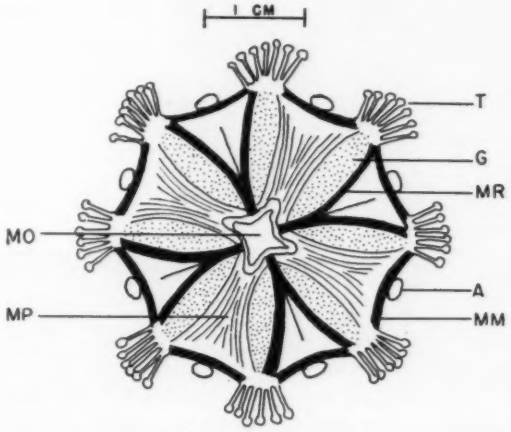

FIGURE 3. Diagram of *H. auricula* subumbrella to illustrate musculature. T, tentacle group; G, gonad; MR, radial muscle; A, anchor; MM, marginal muscle; MO, mouth; MP, perradial muscle.

Spontaneous activity

A considerable amount of apparently spontaneous activity may be observed in *Haliclystus* under both field and laboratory conditions. The arms are flicked in toward the mouth, the calyx is rotated, the stalk alone or the whole animal may contract without any noticeable external stimulation. No well-defined rhythm is apparent from visual observations, nor is any particular order of contracting parts consistently manifested.

Kymograph records of this activity were made on slow drums. There is no orderly progression of height of contraction as in free-swimming scyphozoan jellyfish. The contractions recorded are due primarily to the stalk muscles and do not show the contractions of individual arms. However, such recordings serve to illustrate the arrhythmic nature of spontaneous activity of unmolested animals (Fig. 4, A).

It was apparent from the outset that difficulty would be encountered in attempts to record responses to electrical stimuli in such an active animal. With this in mind, various parts of the body were isolated, to ascertain whether or not any

structure or structures exercised a "pacemaker" control over any other part. Considering the systematic position of the animal, the most obvious place to look for such control was the umbrellar margin, which, in free-swimming scyphomedusae, bears the rhopalia, known to be the seat of pacemaker control of the swimming pulsations. In the majority of medusae (both Hydrozoa and Scyphozoa) removal of the bell margin results in a more or less complete paralysis of the swimming movements (Romanes, 1885; Bullock, 1943). No such pacemaker region has

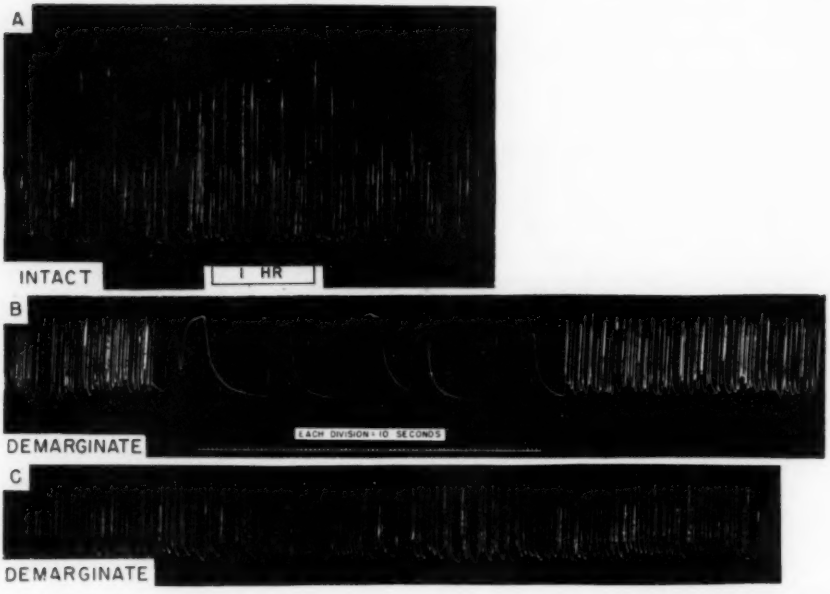

FIGURE 4. Records of spontaneous activity. A. Intact animal. B. Stalk only. Drum speed increased in central portion for 15 minutes. C. Stalk only. Hour marker applies to entire record except central part of B.

been demonstrated in anemones or in hydrozoan polyps, but in the case of anemones, the spontaneous activity is of such a slow nature as to allow investigation of neuromuscular responses with considerable confidence. In *Haliclystus* this activity occurs with sufficient frequency to be extremely troublesome.

In order to determine whether or not "surgical paralysis" was possible, a large *Haliclystus* was set up in such a manner that stalk and calyx contractions could be recorded independently and simultaneously. Pins were placed at the stalk-calyx junction, fastening the animal to a wax-bottomed dish so that the two parts could be separated without disturbing the mechanical recording devices. Contractions of the intact animal were recorded for several hours, after which a sharp knife was drawn across the animal at the stalk-calyx junction, isolating each part from the other, and the recording continued for several more hours. Examination

of the record shows that no difference can be observed in the nature of the contractions before and after separation. Further recordings were made of intact animals and isolated stalks, and no difference was noted (Fig. 4).

Removal of arm tips (tentacles), anchors, bell margin, and, as has been seen, the whole calyx, has no effect on the nature of spontaneous activity. From the above, it is apparent that there is no physiological centralization of the exciting system that manifests any pacemaker control over any other part. The mechanism of this type of activity has not been established directly, but may be inferred from evidence to be presented later.

Responses to mechanical stimulation

The stalk and exumbrella: Stimulation of the surface of the stalk and of the exumbrella in attached and unattached *Haliclystus* seldom elicited a response. Gentle stroking of the exumbrellar surface, stimulation by light touch, and fairly vigorous prodding almost invariably failed to call forth a response. If, however, firm pressure were maintained with the glass rod, *e.g.* on the edge of the pedal disc, a sudden shortening of the stalk and closure of the bell resulted. On several occasions the response was elicited only upon injury to the animal, and continued pressure did not cause a maintained calyx contraction. Shortly after the sudden closure the animal would relax, the calyx relaxing first, even if pressure sufficient to cause injury were maintained. This response could be obtained from any point on the exumbrellar surface and did not appear to be restricted to the pedal disc. The pedal disc does not appear to be any more sensitive than other areas, although the methods used would not give information on any slight gradient of sensitivity. After numerous attempts at mechanical stimulation of the exumbrella, it was concluded that the exumbrellar surface was very nearly insensitive to ordinary mechanical stimulation, responding only to very vigorous stimuli.

This is not surprising when one considers the conditions under which *Haliclystus* normally lives. The population from which experimental animals were taken is almost completely confined to rather dense beds of *Zostera marina,* with the animals usually attached to the blades in a pendant attitude. The *Zostera* is exposed only at the lowest tides, which means that for the greater part of the life-cycle of the animal it is hanging in the water among constantly moving blades of the plant, and is therefore subjected to a considerable amount of buffeting. The exumbrella would be exposed to repeated stimulation by blades of *Zostera,* debris in the surrounding water, and perhaps fairly frequent rubbing over the bottom. If these medusae were very sensitive to exumbrellar stimulation, they would be in the contracted state for a major part of their existence.

Subumbrellar surface and associated structures: The tentacles are extremely sensitive to weak mechanical stimulation. The slightest touch to a single tentacle usually brings about an arm-bending response. Sometimes only a few tentacles react, but more often there is a rapid bending of the whole arm toward the mouth. The arm tips at the base of the tentacles on the exumbrellar side are quite insensitive if the stimulus is applied outside the area of the tentacles, but are quite sensitive on the subumbrellar side.

The entire subumbrella is very sensitive to mechanical stimulation. If the stimulus is applied on the adradius, the arm in the position responds by bending

toward the stimulated spot, and frequently the arm on either side also responds. If the stimulus is applied between the two arms (*i.e.*, in the inter- or perradius), the two adjacent arms respond by bending to the stimulated spot, and frequently, the next adjacent arm on either side also bends. In addition it was noted that the manubrium moved toward the stimulated area whenever the arm-bending response was obtained.

Repeated stimulation of a single spot on the subumbrella may either bring about a total calyx closure, or local insensitivity develops and the animal fails to respond. If a slight stroking movement covering several millimeters is made with a glass rod or silver wire, the total closure response results. It was noted on several occasions that repeated light touches in one spot would bring about total calyx closure in a fairly regular fashion. First the adjacent arms would bend toward the stimulated spot, then on the next stimulus the next adjacent pair, and so on until all eight arms were bent inwards. The exact relationship between stimulus and response in such cases was difficult to assess. If the stimulating object were not removed quickly, it would be caught by the tentacles and the stimulus of this contact would almost invariably cause total calyx closure. Quick removal of the glass rod, however, may have caused sufficient local disturbance to provide additional sources of stimuli, so that accurate appraisal of the stimulus-response relationship under these conditions seems impossible.

If the total calyx closure response were elicited by the stimulus of a linear stroke, stalk contraction and a characteristic rotation of the calyx usually occurred as well. The calyx rotation appeared simultaneously with the stalk contraction.

It was noted that any adequate mechanical stimulus delivered at the edge of the pedal disc always brought about a calyx closure reaction in addition to the stalk contraction, but that adequate calyx (subumbrellar) stimulation did not always elicit stalk contraction. This might be considered evidence of polarization in the conducting system, in the sense that it is "easier" to drive an impulse in one direction than in the other, but due to the methods of stimulation and the extreme difference in the intensity of "adequate" stimulation of the two areas concerned, it would seem unwarranted to accept this observation as evidence of polarity.

Conduction of excitation may be described as diffuse. A single touch elicits the arm-bending response in two to four arms, as well as a shift of the manubrium toward the stimulated point. The so-called "decremental" nature of conduction is also noted in the spread of contraction around the calyx from repeated or more vigorous stimulation. That conduction of excitation is rather slow is quite evident from observations made during mechanical stimulation. The arm nearest the site of stimulation always responds first, the other two following in order depending on whether the stimulated point is nearer one than the other. If they are equidistant they react simultaneously. The reaction is sufficiently slow so that the above is readily observed. Thus, the special properties of the nerve net as outlined by Pantin (1935a) are apparently fulfilled in *Haliclystus*.

Response to electrical stimulation

It has been indicated previously that the normal activity of *H. auricula* presents an unpredictable variable when one attempts to determine the responses of the organism to artificial stimulation. I found no convenient method of suppressing

this activity. Further difficulty was encountered in determining the area to be stimulated. The demonstrated insensitivity of the exumbrellar surface offered the advantage that placement of the electrodes on it caused little disturbance, but this insensitivity may have been the result of absence of nervous elements in the exumbrellar ectoderm. If the electrodes were placed on the subumbrellar surface there was an immediate response to the tactile stimulation, and after a time local insensitivity developed.

The site finally selected was the exumbrella. Kassianow (1901) demonstrated what appears to be a nerve plexus in the exumbrellar ectoderm of Stauromedusae, and experiments to be described support this. Insensitivity to mechanical stimulation may be due to rapid sensory adaptation or to high sensory thresholds and paucity of mechano-receptors in the exumbrella. Neither of these possibilities has been directly demonstrated, but the responses suggest such explanations.

It was necessary to exercise caution in setting up the animals for recording. When possible, the animals were left attached to the *Zostera* on which they were collected and the plant pinned to the bottom of a wax- or clay-lined container. Small glass hooks were used to attach the animal to the recording lever. These were inserted through the bell margin beneath the marginal muscle at one side of an anchor. If the hooks were placed through the arm tips the animals remained unduly agitated for some time, presumably due to stimulation of the tentacles. Each preparation had to be observed carefully to eliminate the possibility of additional stimulation from the recording connections. Animals were not used until three to four hours after having been set up, to allow recovery from the effects of injury. Tests indicated that the responses did not differ appreciably after one-half hour from those after twenty-four hours following injury. All recordings were made from animals in standing sea water.

Response to single-impulse stimulation: As indicated previously, a single condenser-discharge shock results in a contraction of small amplitude in free-swimming Scyphozoa (Bullock, 1943). In the anemones that have been studied (Pantin, 1935a; Hall and Pantin, 1937; Pantin and Vianna Dias, 1952), single-shock stimulation does not result in a recorded response. To determine the response of *Haliclystus* to this type of stimulation, a large specimen was set up for recording and the electrodes applied to the stalk ectoderm.

As might be expected, the results were variable. Stimulation was being applied against a background of spontaneous activity. Contractions were recorded during the course of these experiments, but they were not always associated with the stimulus. Responses occurred before, after, and at stimulation in an apparently random manner. There was no consistent contraction associated with the delivery of the stimulus until high intensities were reached. Examination of preparations receiving such shocks revealed an injured area at the site of the electrodes, and these responses were interpreted as a result of multiple stimuli resulting from injury. Because of the fact that contractions occurred at random with this type of stimulation except at high voltages, it was concluded that *Haliclystus* does not characteristically respond to single-impulse exumbrellar stimulation.

The failure to respond to single-impulse stimulation also characterizes reflex closure of the sphincter of the anemone *Calliactis* (Pantin, 1935a) and the response of the longitudinal mesenteric muscles of *Metridium* (Hall and Pantin, 1937).

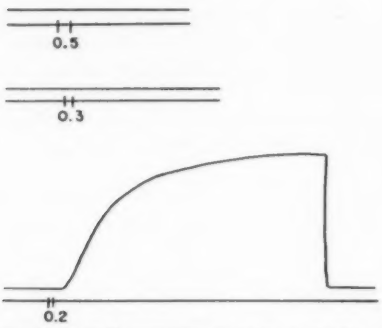

FIGURE 5. Tracings of records of stalk response to pairs of shocks. Upper trace of each pair, contraction record; lower trace, signal marker. Interval between shocks in seconds.

Following single-shock stimulation in *Haliclystus*, a twitching of the tentacles was often observed, as has been reported for *Calliactis* by Pantin (1935a), who considered it an indication that the impulse was being transmitted. In scyphozoan medusae there is a consistent measurable response to a single stimulus, although the magnitude of the response is very much less than that of which the preparation is capable (Bullock, 1943).

Response to pairs of stimuli: After having established the failure of *Haliclystus* to respond in a consistent manner to single-impulse stimulation, the response to pairs of stimuli was investigated. Pairs of shocks at a frequency of one in two seconds, and one, two, three, and five per second were delivered to the preparation. A standard five-minute rest period was observed between the delivery of each pair of stimuli, but because of spontaneous activity this interval was frequently extended. At least one minute of observed inactivity was allowed before the delivery of stimuli. A record of a typical experiment of this sort is shown in Figure 5. It will be noted that a response does not occur until the members of the pair of stimuli are separated by only 0.2 second, *i.e.*, at a frequency of five per second. Occasionally a response occurred at three per second, but the illustrated case is more typical.

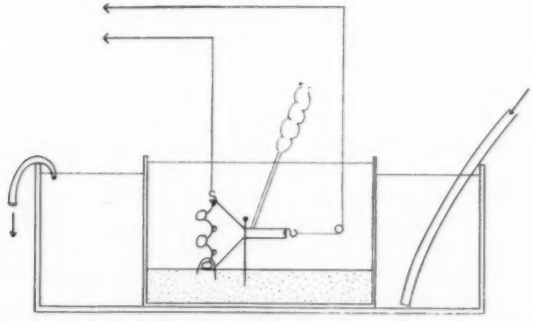

FIGURE 6. Diagram of experimental set-up for simultaneous stalk-calyx recordings.

During the course of the investigation described above, it was noted that total calyx closure regularly occurred at a lower frequency stimulation than stalk contraction. Because of the recording method, such calyx contractions were not recorded. Accordingly, animals were set up in such a way that independent and simultaneous records could be obtained of stalk and calyx contractions (Fig. 6). Stimuli were delivered on the exumbrellar surface at the stalk-calyx junction in the same manner as in the previous experiments. These were repeated on individuals without the encumbrance of the recording and holding devices, and in all cases the results corresponded to those shown in Figure 7. The calyx contracts weakly at a frequency of two per second, more strongly at three and five per second; at the latter interval the stalk contracts, as would be predicted from the previous determination. In contrast to the observed reactions to single stimuli, the responses to pairs of stimuli under the conditions described above are remarkably consistent.

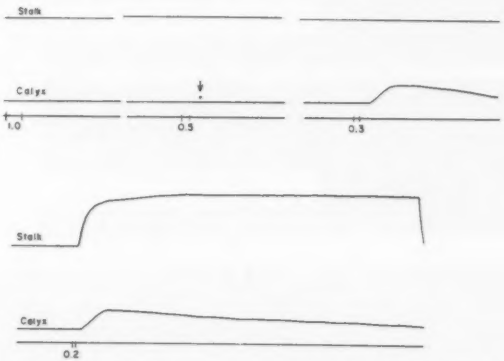

FIGURE 7. Tracings of records obtained as illustrated in Figure 6. Lower trace, signal marker. Interval between shocks in seconds. Unrecorded contraction observed at arrow.

Characteristic response frequencies for different parts of the responding system have been demonstrated in anemones by Pantin (1935b) and can be related to the function of the parts. In *Haliclystus* the calyx is the feeding organ of the animal and as such must be sensitive to very light mechanical disturbances if it is to fulfill its function.

Responses to trains of stimuli: The response to pairs of stimuli is essentially a total response. It is evident on observation of unmolested animals that natural responses are graded, *i.e.*, by no means all responses to naturally occurring stimuli involve all of the responding muscles to the full extent. It is also apparent that each of the three categories of muscles described earlier is capable of at least some independent action. The calyx is able to contract (as has been shown) without stalk contraction, and the stalk under certain conditions may show slow, partial, and frequently asymmetrical responses without the calyx being involved. This is apparent in the characteristic movements by which the expanded calyx is swept through the water, rotated, and inclined at various angles. The marginal muscle is also apparently capable of independent activity, as indicated by certain postures

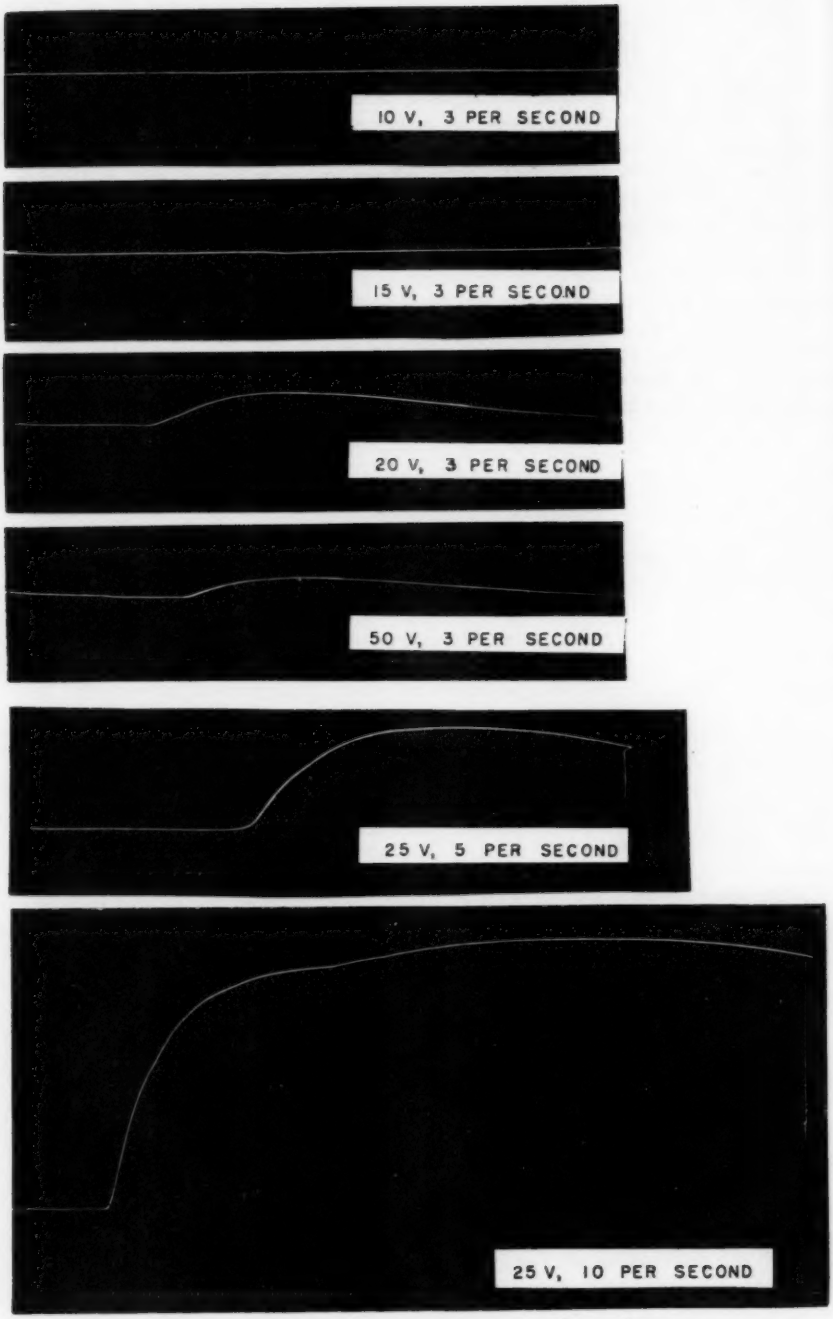

FIGURE 8. Records of stalk responses to various voltages and frequencies of electrical stimulation.

the animal assumes. In the expanded individual it is noted that the eight adradial arms are most frequently equidistantly arranged. At times, however, the arms are drawn together so that they are associated in four pairs, and such pairs may lie in the perradii or, more frequently, in the interradii. Examination of such animals reveals slight folding of the mesoglea over the marginal muscle between the pairs of arms and the corresponding radial muscles of the subumbrella. The intervening muscles show no sign of contraction.

In order to investigate the underlying mechanism of the graded response, the reactions to a variety of stimuli were examined. No information concerning the mechanism of the postural response has been obtained, but certain inferences concerning symmetrical graded responses may be made from reactions to trains of stimuli at different frequencies. Because of the spontaneous activity of the animals, it was impossible to interpret the results when long trains of stimuli at very low frequencies were administered. Any observed contraction could just as well be spontaneous as a reaction to the applied stimuli. At intermediate frequencies, however, *i.e.*, lower than that required for a response to a single pair, but higher than one shock every two seconds, a consistent pattern emerged.

Figure 8 illustrates a possible mechanism of graded response. It will be noted that there is a well-marked threshold and that increasing the intensity of the stimulation does not appreciably affect the height of contraction. At voltages just above threshold, however, increased frequency of stimulation has a marked effect on contraction amplitude.

A similar experiment was conducted on an animal set up for simultaneous stalk and calyx recording, as in Figure 6. The results shown in Figure 9 were recorded from the same animal in the space of less than one-half hour. It will be noted that there is no evidence of a well-marked "staircase" in any of these responses, as has been observed in the anemone *Calliactis* by Pantin (1935a), but that the contractions are essentially continuous. This may be interpreted as the result of the relationship between speed of contraction and decay of facilitation if factors similar to those in other coelenterates are operative. There is none of the machine-like precision in *Haliclystus* that is demonstrated by anemones and free-swimming Scyphozoa. This is probably because of the complicating factors of frequent spontaneous activity, speed of contraction, and the rapid decay of facilitation.

Responses to subumbrellar stimulation: The responses to electrical stimuli reported above are from stimulation of the exumbrellar surface, and are essentially only symmetrical responses. Asymmetrical responses are clearly possible, as indicated by mechanical stimulation and by observation of the undisturbed animal. Certain of these reactions may be examined by subumbrellar electrical stimulation despite the disadvantages pointed out earlier. Such responses have not been recorded because of the weakness of the muscles involved. In some cases, isolated calyces were used, while in others the observations were made on intact animals.

Upon placement of the electrodes there was an immediate and sometimes repeated response, either asymmetrical or total in nature. Electrodes were placed as lightly as possible, but the response ensued upon contact, however light. The animal was then left undisturbed for at least fifteen minutes.

Because of the extreme sensitivity of the subumbrella, precise analysis of the neuromuscular properties was very difficult. Single-impulse stimulation frequently elicited a localized response involving only one or two arms, as was observed follow-

ing light mechanical stimulation. Single-impulse stimulation was never observed to cause a generalized response, but pairs of stimuli at 0.5- to 0.1-second intervals would cause a generalized contraction. Pairs at greater intervals would cause either two localized contractions coincident with stimulus delivery or would spread to the adjacent arms, depending on the interval. If several stimuli were delivered at a frequency of one per second, the contraction would spread in both directions around

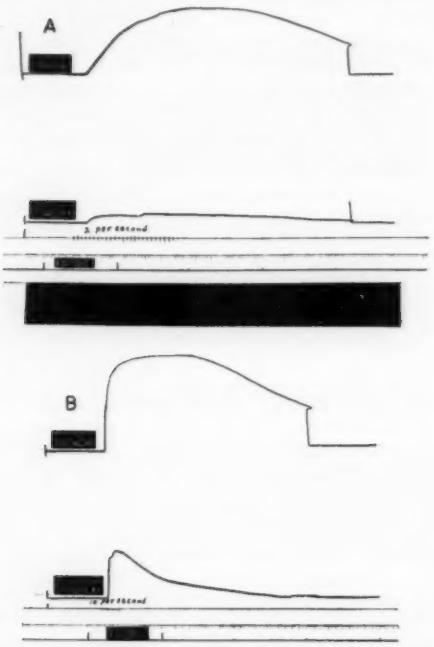

FIGURE 9. Simultaneous stalk-calyx contractions recorded as for Figure 7. Contraction records inked to increase contrast. A. Response to a train of shocks at 2/second. B. Response to a train of shocks at 10/second. Each preparation received 25 shocks.

the calyx, bringing successive arms into contraction coincident with each successive stimulus. Those already contracted would contract again. Thus, the total calyx response could be elicited in a stepwise manner.

A possible explanation of these results is that there is an immediate spread of the impulse to the neuromuscular junctions on the first stimulus and then neuromuscular facilitation governs the excitation of the muscle. A single pair of stimuli at a sufficiently short interval excites the whole system, but it takes several stimuli at lower frequencies to accomplish this. This is probably related to the rapid decay of facilitation, and suggests through-conducting tracts in the subumbrella which, on the basis of this evidence, might be syncytial. Evidence to be presented later, however, suggests that there is interneural facilitation as well.

Conduction speed in the "nerve net"

A number of attempts to measure speed of conduction in the system were carried out. Each determination was repeated several times before any consistency was obtained, and even then there was considerable variation. A strip of tissue was cut from the calyx, leaving one end of this tongue attached to the animal, which was then pinned to a wax-bottomed dish with the stalk projecting upwards. The stalk was connected to a recording lever in the usual way. A pair of stimuli was delivered at an appropriate interval distally on the tongue of tissue, and after five to ten minutes' rest, a similar pair was delivered at a measured distance proximal to the original site. The time from the second stimulus to contraction was carefully measured for each pair of stimuli, and the speed of conduction calculated from the difference in time and the distance between the stimulating electrodes. This was done on several animals, and the values obtained were 7–15 cm./sec. at 11–12° C., comparable to those of 10–20 cm./sec. obtained for *Calliactis* body wall by Pantin (1935b).

Other attempts were made by placing the electrodes at two different points on a line with the reacting muscles on the exumbrella in the intact animal. The results of such determinations varied so radically that they have not been included. Originally the assumption was made in these experiments that no conducting tissue crossed the mesoglea, and that the route of the impulse from stalk stimulation was up the stalk ectoderm, over the bell margin and down the subumbrella to the stalk muscles. The results suggested that there was transmission through the mesoglea at some point along the route.

The form of the "nerve net"

The most careful study of the nervous system of the Stauromedusae is that of Kassianow (1901). In a very thorough investigation using various staining techniques, including vital methylene blue, Kassianow studied the histology and cytology of the nervous system and associated structures in *Lucernaria* (= *Calvadosia*) *campanulata*, *Haliclystus octoradiatus* (= *H. auricula?*), and *Craterlophus tethys*. He describes and figures a diffuse exumbrellar plexus of bi- and tripolar ganglion cells. The experiments to be described largely confirm Kassianow's morphological findings.

Exumbrellar transmission of excitation: In order to test for the presence of exumbrellar nervous elements, several large specimens of *Haliclystus auricula* were selected and a circular incision was made through the stalk ectoderm at a level four to six millimeters above the pedal disc (Fig. 10, A). Following the operation the animals were given two to four hours in which to recover and were then stimulated on the stalk ectoderm aboral to the cut. Under these conditions a contraction could not be elicited.

Stimulus intensities up to 100 volts failed to cause excitation at frequencies at and above threshold for normal animals. Simply transferring the electrodes to the oral side of the cut resulted in excitation at normal frequencies and voltages. Animals were examined for muscle and endodermal continuity before and after stimulation. All animals showed continuity, although there was some damage to stalk canals in all specimens except one which gave the same results as the others.

The stalk of another individual was split from the base up toward the calyx and a cut made from the inside out toward the ectoderm, leaving only the ectoderm in continuity, and stimuli applied basal to the cut (Fig. 10, B). Under these conditions excitation occurred at normal voltages and frequencies. Subsequent examination revealed that a strip of ectoderm two to three millimeters wide was the only continuous tissue except for the very superficial mesoglea underlying the strip. This indicates that conduction of excitation is diffuse. If the conducting tissue is in the form of fibers, these are probably diffuse; but if conduction is a general property of the ectoderm or other superficial layers, one would also expect the results obtained. The latter possibility seems unlikely, but is not excluded by the evidence.

It might then be assumed that the normal path of exumbrellar transmission is over the exumbrellar surface to the bell margin, over the marginal edge to the subumbrellar muscles and to the stalk muscles. To test this assumption, the entire subumbrellar surface, including the oral region, was removed from an animal (Fig. 10, C). The ring muscle was left intact, as were the distal ends of the gonads, the arm tips, and tentacles. Under these conditions there is no ectodermal continuity from any point on the exumbrella to the stalk muscles, and since the radial muscles of the calyx have also been removed, transmission cannot take place through them. Stimuli were then delivered at the base of the stalk, and at normal voltages and frequencies a response of the stalk muscles was elicited. Such a preparation does not respond to a pair of stimuli at 0.2-second interval as does an intact animal, which may indicate that the normal pathway of excitation has been interrupted. Also, this preparation still had some septal tissue, which may have given continuity.

To insure no marginal continuity, another animal had the entire upper portion of the calyx removed as well as the subumbrellar tissue (Fig. 10, D). Under these conditions, a contraction could also be obtained at normal voltages and frequencies, although it took four shocks at five per second to elicit the response.

Still another animal was completely demarginated, the stalk split from the base up to within one millimeter of the cut edge, and stimuli applied at the base of one-half of the stalk (Fig. 10, E). This meant that if excitation were transmitted to the other half, it would be forced to traverse the mesoglea at some point in the non-stimulated half after having gone through the one-millimeter strip of ectoderm at the cut margin. The four groups of stalk muscles are separated by mesoglea at this level, and there is no opportunity for transmission through the split base of the stalk. Under these conditions, the non-stimulated half responds to shocks at normal voltages and frequencies. In this preparation there was some continuous endodermal tissue at the oral end, but the previous demonstration of the necessity of ectodermal continuity for transmission in the stalk reduces the significance of this. Furthermore, it does not affect the contention of mesogleal transmission, because neither the endoderm nor the ectoderm is in contact with the muscles.

Wietrzykowski (1912) clearly shows the development of the stalk muscles from the "cordon cellulaire de la taeniole" which has its point of proliferation in a subumbrellar position. This means that the direct innervation of the stalk musculature from the exumbrellar ectoderm is a result of conducting tissue arising in the exumbrellar ectoderm and becoming associated with the musculature, or of con-

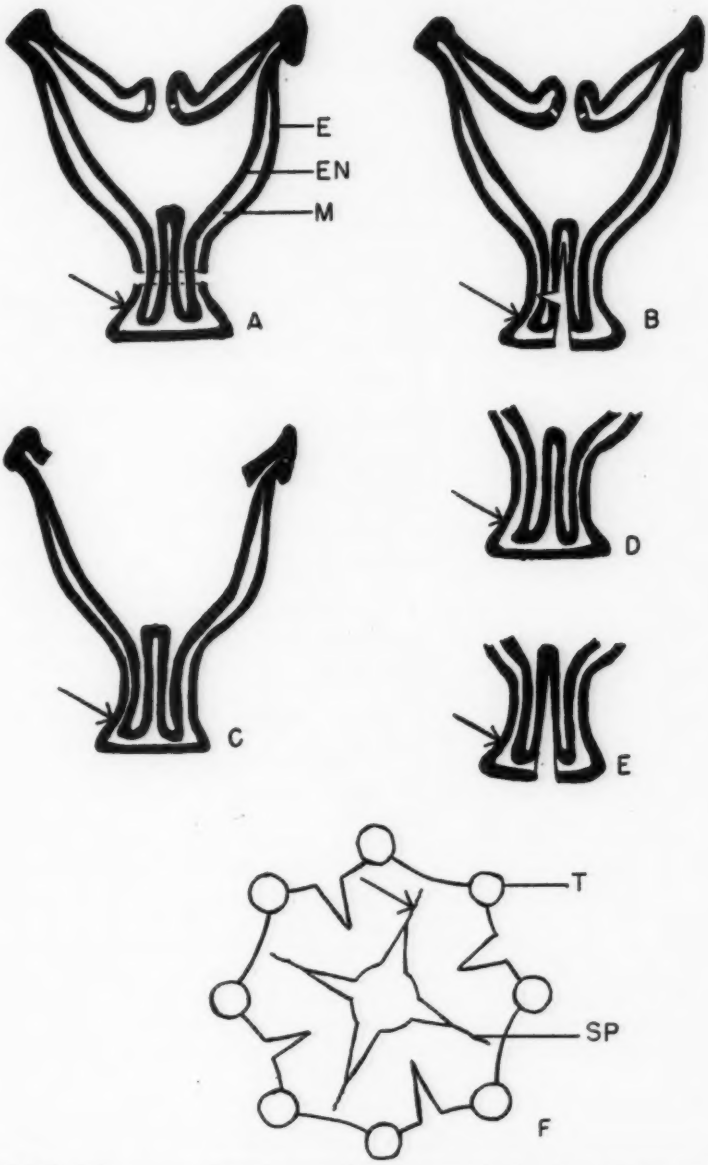

FIGURE 10. Diagrams to illustrate operations to ascertain the form of the conducting system. See text for explanation. E, ectoderm; EN, endoderm; M, mesoglea; SP, septum; T, tentacles. Position of stimulating electrodes indicated by arrows.

ducting tissue migrating out from the muscle to the ectoderm. Horridge (1956), however, has demonstrated a diffuse nerve net on the exumbrellar surface of the ephyra of *Aurellia aurita*, and it is possible that the exumbrellar conducting system of *H. auricula* is the homologue of the diffuse system in other scyphozoans.

Subumbrellar transmission of excitation: To ascertain the nature of subumbrellar conduction, the stalk and oral structures were removed from a specimen of *Haliclystus*. Four incomplete cuts were made from the margin to within a few millimeters of the cut inner surface between alternate pairs of arms, and similar cuts were made from the inside out toward the margin, alternating with the first cuts described (Fig. 10, F). This gave a ring preparation similar to those used by Romanes (1885) in which the impulse is forced to take a devious path to circle the bell. The electrodes were applied in the position indicated and trains of impulses were delivered to the preparation.

Under these conditions a train of impulses at normal voltage and at a frequency of five per second elicited a local contraction involving only the nearest two or four arms. At ten per second, the whole calyx could be brought into contraction in the following particular manner. First the arms nearest the electrodes respond, then those on each side in sequence and by pairs until all contract. There is a noticeable delay between the contractions of successive parts, as if junctions were being facilitated in turn. This preparation emphasizes the functional autonomy noticed in the intact animal. It also establishes the diffuseness of conduction and proves that no one pathway is essential for conduction around the bell. The expected pathway would be around the bell margin in the region of the marginal muscle, but the preparation described above shows that continuity of the muscle, and hence of any accompanying nerve tracts, is not essential to conduction. With arms and anchors removed, the preparation reacts in the same way as when these structures are present.

The above indicates that the arms are functionally associated into pairs. This functional pairing appears to depend on the integrity of the intervening marginal muscle and may indicate a normal through-conducting mechanism around the bell from arm to arm. Furthermore, if the stimulus is applied in the interradius, the contraction progresses by interradial pairs of arms. If applied in the perradius, the first reaction involves the adjacent arms only, or may involve the interradial *pair* on either side. This indicates that an interradial pair of arms, with its associated radial musculature (see description of musculature) and marginal muscle, has a degree of autonomy as a pair that separates it functionally from the other interradial pairs.

If the above results are examined in the light of stimulation of the intact calyx, the evidence for a through-conducting system is supported. That it is probably associated with the marginal muscle is indicated by the failure to respond to a pair of stimuli when this muscle is cut. It appears that under these conditions of mutilation, several junctions must be facilitated in order to bring about a generalized contraction.

Discussion

The results of this study enable one to construct a coherent picture of the normal behavior and responses of *Haliclystus auricula*. The pulsating contractions of the free-swimming Scyphozoa are lacking in *Haliclystus*. It has been demonstrated that there is no pacemaker present, and also that the requisite degree of specialization

of the conducting system is lacking. Thus, the calyx of *Haliclystus* lacks the capability of responding in the repetitive fashion of its free-swimming relatives to repeated shocks at regular intervals. However, the available evidence indicates that a through-conducting system is normally operative in the subumbrella of the calyx. No evidence for higher conduction speed in that part of the animal, as compared to other parts, has been obtained. The evidence is primarily the different responses of experimentally incised and intact animals. The through-conducting system is brought into play only by relatively high frequency stimulation. At low frequencies the radial parts of the calyx exhibit considerable autonomy. In addition to this through-conducting system there also appears to be a diffuse "nerve net" which operates to correlate the subumbrellar structures, such as the manubrium, with the action of the principal food-gathering devices, the arms and the tentacles. There is no clear evidence of more than one nerve net as has been reported in *Aurellia* by Horridge (1956).

Inherent spontaneous activity may be interpreted in the following way: no part of the animal exercises control over any other part. From this we may assume that impulses are spontaneously generated in many, if not all, ganglion cells. Such impulses spread over the entire nerve net, but do not activate the muscles concerned unless two or more arrive at any one junction within an appropriate time interval. The nature of the spontaneous activity seems to indicate that each ganglion cell discharges at its own rate, each independently of the others. Activity may or may not be rhythmical. The graded and asymmetrical nature of the spontaneous activity, together with the lack of a regular pattern supports such an explanation. A pattern of endogenous activation of the muscles could lead to the observed sweeping, "seeking" motions of the unstimulated animal. Exogenous stimuli impinging upon the exumbrella must be of sufficient intensity to generate relatively high-frequency impulses if the protective closure-contraction response is to ensue, as indicated by the relatively high frequency of electrical stimulation required to bring about a response. The subumbrella, on the other hand, responds as a whole to lower frequency stimulation in a local fashion. Thus, if a food organism brushes very lightly over the subumbrellar surface a suitable reaction will be forthcoming in the arm-bending response to secure the prey. If the prey struggles, further impulses are generated, and the entire calyx responds to ensure capture and ingestion. The role of chemical stimuli in the predation process has not been investigated, but is no doubt of some importance—especially in the discharge of nematocysts and gland cell secretions.

If, as has been assumed, *Haliclystus* possesses a simple plexiform nerve net, the reactions reported in this study are subject to two possible interpretations. The high degree of autonomy noted in different regions of the subumbrellar structures is most easily explained on the basis of neuroneural facilitation at synapses between strategic areas of the net, or a hierarchy of thresholds of neuromuscular junctions to frequency and number of arriving impulses. Such a system, along with the endogenous activation of muscles, is admirably adapted to sweeping through the water in a "seeking" motion, and to catching prey on contact.

The exumbrellar nerve net shows a high degree of facilitation with rapid decay characteristics not noted in either anemones or free-swimming Scyphozoa, and this occurs at comparatively low temperatures (11–13° C.). The relationship of decay

of facilitation (rapid) to contraction time (comparatively rapid) to relaxation time (relatively slow) is such that a fused contraction results at frequencies sufficiently high to elicit a contraction.

The subumbrellar nerve net exhibits the properties of through-conduction, neuromuscular facilitation and, probably, interneural facilitation. It responds locally to single-impulse stimulation, indicating a partial permanently facilitated state.

The properties of the neuromuscular system of *Haliclystus* do not appear to differ in any fundamental way from those noted in anemones (Pantin, 1935a, 1935d) and free-swimming Scyphozoa (Bullock, 1943). Modifications of the characteristics of the nerve net with regard to the time sequence of decay of facilitation and certain time characteristics of the muscles would appear to be sufficient to explain the differences in behavior noted in these animals. No new properties need be postulated to account for the reactions that do not fall in the range of variation already noted in the coelenterates.

Bearing in mind the presumed derivation of the Stauromedusae from free-swimming Scyphozoa, we may speculate upon the differences involved with the sessile habit. The most obvious of these is the loss of the pacemaker mechanism of swimming, and the loss of (or failure to develop) the specialized through-conducting mechanism that makes the swimming pulsations possible. Secondly, the stalk muscles are able to maintain a prolonged contraction not possible in free-swimming Scyphozoa. This is not as pronounced in the calyx musculature, which relaxes rather quickly even under sustained stimulation (see Figure 9). The properties of the mesoglea indicate that contraction is maintained against a mesogleal restoring force, hence the muscle must possess a refractory period of shorter duration than that found in free-swimming forms. Since sustained contraction differs in the stalk and calyx muscle systems, it is probable that each system has a muscle refractory period different from the other. Neither system is able to maintain a contraction for the length of time noted in anemones, so it may be reasoned that the physiological properties of both systems lie somewhere between those of free-swimming scyphozoans and those of anemones.

Summary

1. Previous work on the neuromuscular systems of coelenterates has been confined to Anthozoa and free-swimming medusae, and certain differences in the response to electrical stimulation have been noted. It was thus of interest to investigate the responses of a sessile scyphozoan, *Haliclystus auricula* (Rathke), to determine the characteristics of the response mechanism.

2. Spontaneous activity of *H. auricula* is arrhythmic, and there is no demonstrable "pacemaker." The exumbrellar surface of the organism is relatively insensitive to mechanical stimuli, but the tentacles and subumbrellar surfaces are very sensitive. The conducting system possesses properties of a "nerve net." Conduction is slow, diffuse, and with an apparent decrement.

3. *H. auricula* does not respond to single-impulse electrical stimulation of the exumbrella. A pair of stimuli elicits stalk contraction at an interval of 0.2 second, and calyx contraction at 0.5 second. On the other hand, single-impulse stimulation of the subumbrella will elicit a response, but this response is local, involving only a part of the calyx near the position of the electrodes. A possible mechanism of the graded response is suggested.

4. Physiological evidence indicates that the conducting system is diffuse, with some evidence of a through-conducting pathway at the bell margin that operates at relatively high-frequency stimulation. Conduction speed in the subumbrellar nerve net is calculated at 7–15 cm. per second at 11–13° C.

5. The physiological properties of the neuromuscular system of *Haliclystus* appear to be intermediate between *Calliactis* and *Cyanea*. There is a demonstrable difference in the response of stalk and calyx which shows emphasis on sequential elicitation by mechanical stimulation of progressively greater intensity of tentacle, local marginal, essentially whole calyx, and finally symmetrical stalk involvement. The significance of this series of events in relation to the normal behavior of the animal is pointed out.

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SEASONAL CHANGES IN COLD-HARDINESS OF FUCUS VESICULOSUS

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Some marine algae, such as the fucoids, grow attached above mean low water and are, therefore, subjected at ebb tide to rather severe atmospheric conditions in some climates. In winter in the arctic, species of *Fucus* may be subjected to air temperatures down to − 40° C., yet do not seem to be at all injured (Scholander, Flagg, Hock and Irving, 1953; Kanwisher, 1957). The drying resistance of marine algae has been studied frequently in the past and it is apparent that those growing highest in the littoral zone are generally the most desiccation-resistant (Muenscher, 1915; Pringsheim, 1923; Isaac, 1935; Stocker and Holdheide, 1937; Biebl, 1939). The fact that they are desiccation-resistant suggests that they might also be fairly cold-resistant. This idea is borne out by findings of Kylin (1917) with *Fucus* and by those of Biebl (1958) with various marine algae.

In spite of this work, there appear to have been no seasonal studies of cold-hardiness of the common littoral marine algae. A number of such studies have been made with land plants (for example, Parker, 1955, 1959), and these have shown that there are wide fluctuations in cold-hardiness with the season in all woody plants of relatively cold climates. It therefore seemed of general biological interest to determine whether a marine plant such as *Fucus vesiculosus* L. would also go through such fluctuations.

Methods

From June of 1958 to late May of 1959 *Fucus* plants were collected from convenient locations along the Long Island shore, near New Haven, as a preliminary study. Although there appeared to be a definite trend in hardiness from about − 20° C. in August to about − 60° C. in February, results were so irregular that it was suspected that some other environmental factor or factors besides air temperature were involved. It was therefore decided to repeat these experiments another year but to obtain the plants from a single location, both as regards the harbor and the position above low tide. It was found that hardiness varied as much as 15° C. in May between plants brought from about the mean low tide level and those brought from the highest fucoid zone, the higher ones being more resistant. This latter zone was used in the 1959–1960 experiments which are reported in this paper. It was decided that the stone jetty at Hammonasset beach, near Madison, Connecticut, was the best location, since it was accessible and the water relatively clean. An attempt was also made to obtain the plants as near to 2:00 P.M. as possible, in spite of variable tide levels for a particular time of day at different times of the year.

Fucus plants about eight inches long were plucked from the rock, put in

stoppered jars of fresh sea water, and taken to the laboratory within 30 minutes. Fronds three inches long, including reproductive tips as well as vegetative ones, were cut from these plants, blotted on filter paper, and put in 500-ml. Dewar flasks in a damp condition. These were stoppered with a cork and cooled by an ethane compression system in a low temperature apparatus having a two-foot square compartment (Parker, 1959). The ethane, in turn, was cooled by a Freon-22 compressor system (Cincinnati Sub-Zero Products, Ohio). After being cooled at a rate of

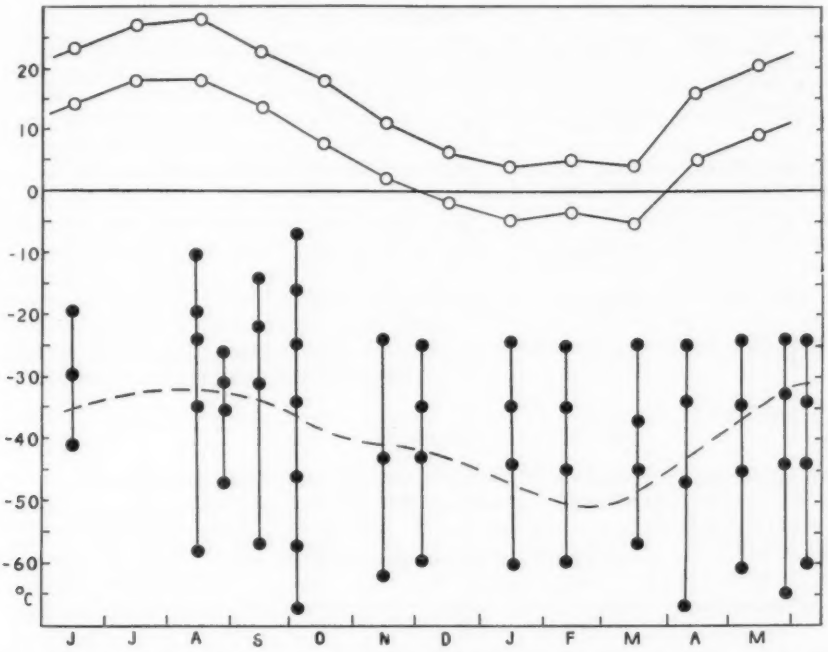

FIGURE 1. Upper curves represent maximum and minimum temperatures at the New Haven municipal airport, averaged for each month for the 1959–1960 season. Lower curve (dashed) represents the relative hardiness of *Fucus*, drawn rather freely as the approximate point at which 50% of the frond was alive (reducing tetrazolium chloride). Data are given in Table I. Blacked-in circles represent temperatures to which a group of plants were cooled on a particular day.

4° C. per hour to one of the temperatures indicated by the blacked-in circles in the results (Fig. 1), flasks were removed to an ordinary refrigerator for three hours and then to room air to obtain a warming rate of about 8° C. per hour.

Determination of viability by ordinary means, for example by leaf color, is very difficult in these marine algae and may account for the lack of research in this field. But by means of the tetrazolium test, very clear and reliable results could be obtained. The fact that reduction of this compound by dehydrogenases to its red formazan derivative is a good indication of cellular viability has been previously

discussed (Parker, 1953a, 1953b). One-inch-long cuttings from the tops of the cold-treated frond material were placed in 8-ml. test tubes and 6 ml. of a solution added, consisting of a 1 : 1 mixture of sea water and 0.6% 2,3,5-triphenyl tetrazolium chloride in tap water. Tubes were stoppered and placed in the dark for 4 to 18 hours at 23° C. Although some results can be observed in 4 hours, it was found best to wait 18 hours. After about 24 hours, bacterial action commonly interferes with results and clouds up the water with red precipitate. Reliability of the tetra-

TABLE I

Data shown in the lower part of Figure 1, together with effects of tetrazolium (TTC) test

June 15	Temp.*	−19	−29	−42				
	TTC test**	100	100	0				
Aug. 15	Temp.	−10	−19	−24	−36	−58		
	TTC test	100	100	100	40	0		
Aug. 29	Temp.	−26	−31	−36	−48			
	TTC test	100	50	40	5			
Sept. 10	Temp.	−14	−23	−32	−57			
	TTC test	100	100	40	0			
Oct. 5	Temp.	−8	−17	−25	−34	−46	−57	−67
	TTC test	100	100	100	100	5	0	0
Nov. 15	Temp.	−24	−43	−62				
	TTC test	100	5	0				
Dec. 4	Temp.	−25	−35	−42	−59			
	TTC test	100	100	10	5			
Jan. 15	Temp.	−24	−35	−43	−59			
	TTC test	100	100	80	10			
Feb. 12	Temp.	−25	−35	−44	−59			
	TTC test	100	100	80	15			
Mar. 18	Temp.	−25	−37	−44	−57			
	TTC test	100	100	60	50			
Apr. 10	Temp.	−25	−35	−47	−67			
	TTC test	95	100	10	5			
May 9	Temp.	−24	−35	−45	−61			
	TTC test	100	70	5	3			
May 31	Temp.	−24	−32	−44	−65			
	TTC test	100	5	0	0			
June 6	Temp.	−24	−34	−44	−60			
	TTC test	100	5	0	0			

* Temperature in degrees centigrade to which fronds were cooled.
** Frond surface in % showing positive tetrazolium test.

zolium test was also supported by the fact that *Fucus* failing to reduce the compound emitted a rotten odor in 48 hours while those reducing it did not.

Results

Cold-hardiness was at a minimum during the summer at about − 30° C. (Fig. 1). Hardiness increased by October and by late winter reached about − 50° C. Since there were frequently borderline cases of damage, it was necessary to express results in terms of percentage tissue area reducing tetrazolium chloride (Table I). The dashed line in Figure 1 is drawn at approximately the point where there was 50% of the tissue not injured (reducing tetrazolium chloride). But even this system was somewhat complicated by the fact that in late summer, growing tips were more sensitive to cold than the rest of the plant, whereas in winter and early spring, tips appeared to be more hardy than the rest of the plant.

In spring, dehardening began to appear by mid-April when woody land plants were showing rapid dehardening. This was apparently related to the higher air temperatures of spring, beginning near the end of March. Surface water temperatures measured at the western end of Long Island Sound (Anon., 1947) and plotted as monthly means for a year showed little deviation from the air temperature data shown in Figure 1. It is therefore impossible to say whether air or water temperature is better related to changes in hardiness. There is, of course, no proof that seasonal changes in hardiness are not controlled endogenously or by some other environmental factor such as day-length.

Summary

A seasonal study of the changes in cold-hardiness of *Fucus vesiculosus* L. was made over a two-year period. Plants in summer could withstand about − 30° C. (the lowest temperature at which 50% of the frond was still alive after treatment), whereas in January and February, plants could withstand − 45° C. to nearly − 60° C. Changes in hardiness with the season appeared to be related to air temperature variations, but also to mean monthly surface water temperatures plotted for a year. In May, *Fucus* taken from the low tide level was 15° C. more sensitive to the cold treatment than those from the highest level of the fucoid zone. Growing tips were the hardiest part of the plants in winter and early spring, but the least hardy in late summer.

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STUDIES ON MARINE BRYOZOA.

XIII. TWO NEW GENERA AND NEW SPECIES FROM ANTARCTICA

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The purpose of the present study is to describe two new genera, *Toretocheilum* and *Isoschizoporella*, and a new species, *T. absidatum*, from Marguerite Bay, Antarctica, and to elevate a previously known variety, *Schizoporella tumida* var. *tricuspis* Calvet 1909, to species rank: *Isoschizoporella tricuspis* (Calvet) 1909. The two species were collected for the Smithsonian Institution by the U. S. Navy's 1947–1948 Antarctic Expedition (hereafter referred to as USN) by Comdr. D. C. Nutt. The writer wishes to express her very grateful appreciation to the Smithsonian, U .S. National Museum, for the loan of these specimens and to the National Science Foundation for grants so generously supporting this and related researches.

Both species belong to the family Hippoporinidae as redefined by Osburn (1952, pp. 316 and 343). He limited the family to include those schizoporellid species which have a pleurocyst or olocyst type of frontal wall with marginal areolar pores (areolae), avicularia, usually strong cardelles, orifice and operculum constricted at sides, in some species at least.

GENUS *TORETOCHEILUM*, NEW GENUS

Diagnosis. Colony encrusting. Zooecia entirely adherent. Dietellae present (Fig. 9). Zooecial front wall convex, an areolate and moderately costate pleurocyst. Frontal not otherwise porous. Large pointed adventitious avicularia on front, over areolar pores (Fig. 1). Globose ovicell with areolae around border in ectooecium or where ectooecium and entooecium meet (Figs. 2, 3, 5). Ovicell partly immersed in next distal zooecium (Fig. 5). Zooecial operculum does not close ovicell aperture. Low spine-bearing peristome surrounds zooecial orifice (Figs. 1, 6, 10). Orifice suborbicular, laterally and distally bounded by a C-shaped vestibular arch which supports the operculum (Figs. 6, 7). Vestibular arch ends proximally on each side in thick ledge-like cardelle (Figs. 8, 10). Between the cardelles is a gap or inner sinus closed by the tab of the operculum (Fig. 8). In front of this, externally, the low peristome rises to form two curved cusps which encircle the rounded median peristomial sinus (Fig. 10).

Name derivation. The genus was named *Toretocheilum*, meaning "pierced lip," because of the sinus-pierced proximal peristome which forms the lower "lip" of the orifice. It is of Greek derivation (see Brown's lexicon) from *toretos*, bored or pierced, and *cheilos* (neuter noun), lip or rim.

Type species: Toretocheilum absidatum, n. sp.

Type locality: Marguerite Bay, Antarctica, Sta. 240.

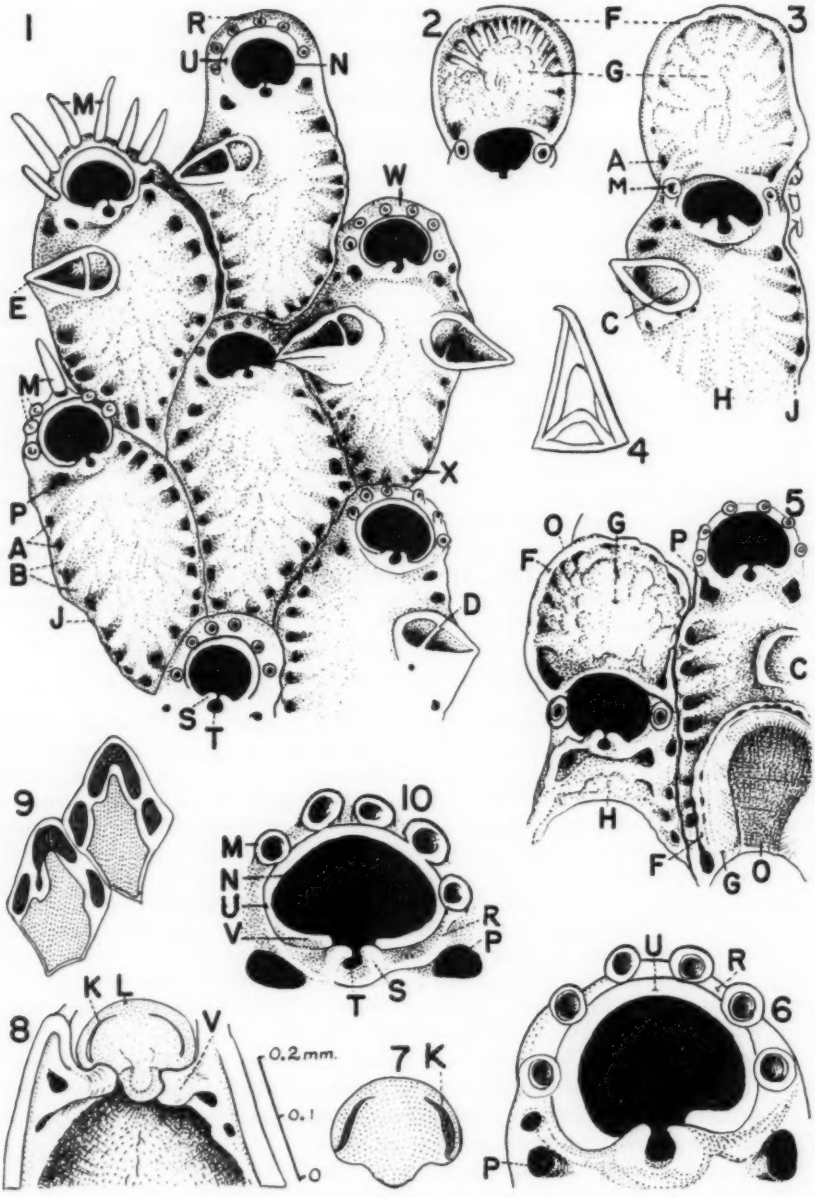
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PLATE I.

Remarks. The new species *absidatum* presented a problem in identification and relationships. It had features strongly allying it to both genus *Chiastosella* and genus *Stephanosella.*

Chiastosella is known only from the southern hemisphere and is represented by both fossil and Recent species. *Stephanosella* has a wider distribution.

Chiastosella is classified in different families by different authors. Stach (1937) and Brown (1952) allocate it to the family Schizoporellidae, while Bassler (1953) allocates it to the family Hippoporinidae, which was once a subfamily of Schizoporellidae but which is now a separate family. Bassler (1953) and Osburn (1952)

Toretocheilum absidatum, new species

List of Abbreviations Used on the Plates

A Areolae or areolar pores
B Areolar costae
C Avicularial chamber
D Avicularial pivot
E Avicularium
F Ectooecium of ovicell
G Entooecium of ovicell
H Frontal wall
J Mural rim
K Opercular sclerite
L Operculum
M Spines or spine bases
N Orifice of zooecium
O Ovicell
P Paraoral areolar pores
R Peristome
S Peristomial cusps
T Peristomial sinus
U Vestibular arch or collar
V Vestibular ledge or cardelles
W Distal part of zooecium
X Proximal part of zooecium
Y Distal wall
Z Lateral wall

PLATE I

All figures on this plate are of *Toretocheilum absidatum,* new genus and new species, and are drawn with the aid of a camera lucida.

Figure 1. Seven calcined non-ovicelled zooecia, some with one or two avicularia, some without any.

Figure 2. An ovicell drawn directly from the rock under low power (dissecting microscope). Above the two spine bases are the spaces ("pores") between ectooecium and entooecium.

Figure 3. A calcined ovicelled zooecium with an avicularial chamber in which the underlying areolar pore is visible. The floor of the avicularial chamber is the frontal wall of the zooecium.

Figure 4. Mandible of an avicularium.

Figure 5. Two young calcined zooecia, the one at left ovicelled, the one at right non-ovicelled. At lower right, imbedded in the proximal front wall of the latter, is a damaged ovicell which belongs to a zooecium below those shown. The front wall of the damaged ovicell is broken off, exposing the inner wall of the ovicell. The ovicell rim shown over the orifice later becomes reduced as calcification proceeds.

Figure 6. External view of orifice of a non-ovicelled zoid. One cardelle of vestibular arch is hidden by a peristomial cusp.

Figure 7. Operculum with curved sclerites for muscle attachment. The median tab below fits the space between the cardelles and the back of the cusps.

Figure 8. Interior of the frontal wall, operculum and half the orifice. The thick ends (V, ledges or cardelles) of the vestibular arch hold the operculum in place. Through the opercular tab can be seen the peristomial cusps and peristomial sinus. These peristomial structures are external to the operculum. Drawn to the 0.2 mm.-long scale at right.

Figure 9. The attached back or basal wall of two zooecia, showing the three distal darkly shaded dietellae of each.

Figure 10. Zooecial orifice tipped forward to show the exact relation of the C-shaped vestibular arch and vestibular sinus to the peristomial cusps and peristomial sinus.

place *Stephanosella* in the family Hippoporinidae. The difference between the two families is based on the nature of the orifice and the frontal wall.

The new USN species *absidatum* is closer to *Chiastosella daedala* (MacGillivray) 1887, type species of *Chiastosella* Canu and Bassler 1934 (see Bassler, 1934, p. 407) as regards the distinctive orifice, peristome, spines, and avicularia but differs in the type of frontal wall and ovicell porosity and sculpturing. In the latter features (frontal wall and ovicell) *absidatum* is closer to *Stephanosella* Canu and Bassler 1917, but the orifices are quite different.

Chiastosella at present contains a diversity of species (*cf.* Stach, 1937; D. A. Brown, 1952, 1954) that could split the genus between two families (Osburn's limited families Hippoporinidae and Schizoporellidae). Therefore, it was thought more sensible to erect a new genus *Toretocheilum* for the dissident and complicating USN *absidatum* than to further diversify the genus *Chiastosella* by including a new species of such divergence from the type *C. daedala*.

Diagnosis. Colony encrusting, well calcified. Zooecia convex and approximately hexagonal. Frontal a pleurocyst with one row of areolae, the paraoral pair often more pronounced. Areolar grooves and costae prominent. None, one or two large transverse, pointed avicularia over areolar pores on mid-frontal corners. Five to 7 stout oral spines on peristome. Orifice suborbicular, lined by a C-shaped vestibular arch whose ends form prominent blunt cardelles proximally. Proximal border of orifice straight to concave, with round, median sinus inserted between or flanked by two arched cusps. Ovicell with row of areolae where ectooecium and entooecium meet peripherally, but otherwise non-porous. Ovicell has faint proximal rim, bordered by two oral spines. Three or more large basal dietellae.

Name derivation. The species *T. absidatum* was named for the arched proximal peristomial cusps which outline the median orificial sinus, and also for the remainder of the arched orifice. The trivial name is derived from the Latin *apsis* (*absis*), arch; *absidatus*, arched, vaulted. (*Cf.* Brown's lexicon.)

Measurements. Given below are minimum, maximum and the average of a number of readings, usually 10, in millimeters. L is for length, W for width, D for diameter.

0.965–1.267	(1.081)	L	zooecia
0.432–0.763	(0.634)	W	zooecia
0.576–0.605	(0.586)	L	ovicell, 4 readings
0.547–0.576	(0.566)	W	ovicell, 4 readings
0.202–0.245	(0.217)	L	orifice, including sinus
0.216–0.245	(0.230)	W	orifice
0.014–0.058	(0.033)	D	sinus
0.259–0.331	(0.297)	L	avicularium
0.130–0.173	(0.151)	W	avicularium
0.216–0.346	(0.250)	L	oral spines, 6 readings
0.029–0.058	(0.045)	D	oral spines
0.163–0.202	(0.182)	L	mandible, 4 readings
0.124–0.130	(0.128)	W	mandible, 4 readings

Colony. Four small patches of colony were found encrusting a thick flat hand-sized rock from Marguerite Bay. Pieces had to be scraped or burned off for study

because they were on the sides of the rock in areas that could not be maneuvered for study under the compound microscope.

Zooecia. Zooecia are box-like, their fronts 4- to 6-sided. The side walls are vertical, low and about the same height all around. Three or so large dietellae outline the distal half of the basal wall (Fig. 9).

The frontal wall is very convex, mound-like, with thin, raised mural rim. A row of deeply sunk oval areolae outlines it. Grooves radiate centerward from the areolae. Short costal ridges separate them.

The frontal wall consists of a translucent calcareous olocyst fast overgrown by an opaque granular or roughened pleurocyst. The central part of the pleurocyst is non-porous except for an occasional pore puncturing the avicularial chamber near its base. The areolar pores are not large but the grooves and ridges make them more conspicuous than would otherwise be the case. The two areolae nearest the orifice corners are often slightly larger than the others (Figs. 5, 6). In young, less heavily calcified zooecia the grooves leading from these two paraorificial areolae pass just proximal to the orifice, leaving the orifice elevated slightly above the immediate frontal wall (Fig. 5).

Avicularia. Avicularia are all of one type and of approximately the same size. Usually one, sometimes two, large pointed avicularia occur on some zooecia. Other zooecia may be without any. If two avicularia are present on one zoid they are placed bilaterally, opposite each other. Avicularia are located at the corners over areolar pores, midway up the side of the front wall. They are transversely oriented with mandible pointing out. Sometimes they slant a bit obliquely (Figs. 1, 3).

Each avicularium is mounted on a prominent avicularial chamber which tips the avicularial beak to a slightly upward-directed or oblique position. The avicularial chamber is large at the base and covers one or two areolae. As is the custom in calcified species with large adventitious avicularial chambers, there is an occasional small pore perforating the chamber wall near its base, probably for hydrostatic reasons.

The avicularial back area is hemispherical. The beak is pointed. Separating them is a straight pivot bar on which the base of the triangular mandible rests. The mandible is a tall narrow triangle, with curved tip and a lucida near its base (Fig. 4).

Orifice. The unusual orifice of *T. absidatum* made it difficult to classify because either few species have this type or else if they have it the illustrations generally do not show the orifice in very great detail.

The orifice is tipped forward a bit distally so that it seems horizontal. In front of it the frontal wall soon rises like a mound. The orifice is placed at the very extremity of the frontal wall.

It is lined by a C-shaped inner collar, the vestibular arch, whose ends curve around proximally to form the cardelles (Figs. 8, 10). The cardelles are sturdy, blunt, smooth usually and separated from each other by a median gap approximately one-third the width of the orifice.

In front of the gap is the rounded spout-like sinus whose walls are formed by the two curving cusps which arise from the proximal wall of the peristome (Figs. 6, 8, 10). The sinus and gap form an inward slanting channel. In young zooecia the orifice with the frontal peristomial tabs and sinus is set off from the zooecial

Isoschizoporella tricuspis (Calvet) 1909

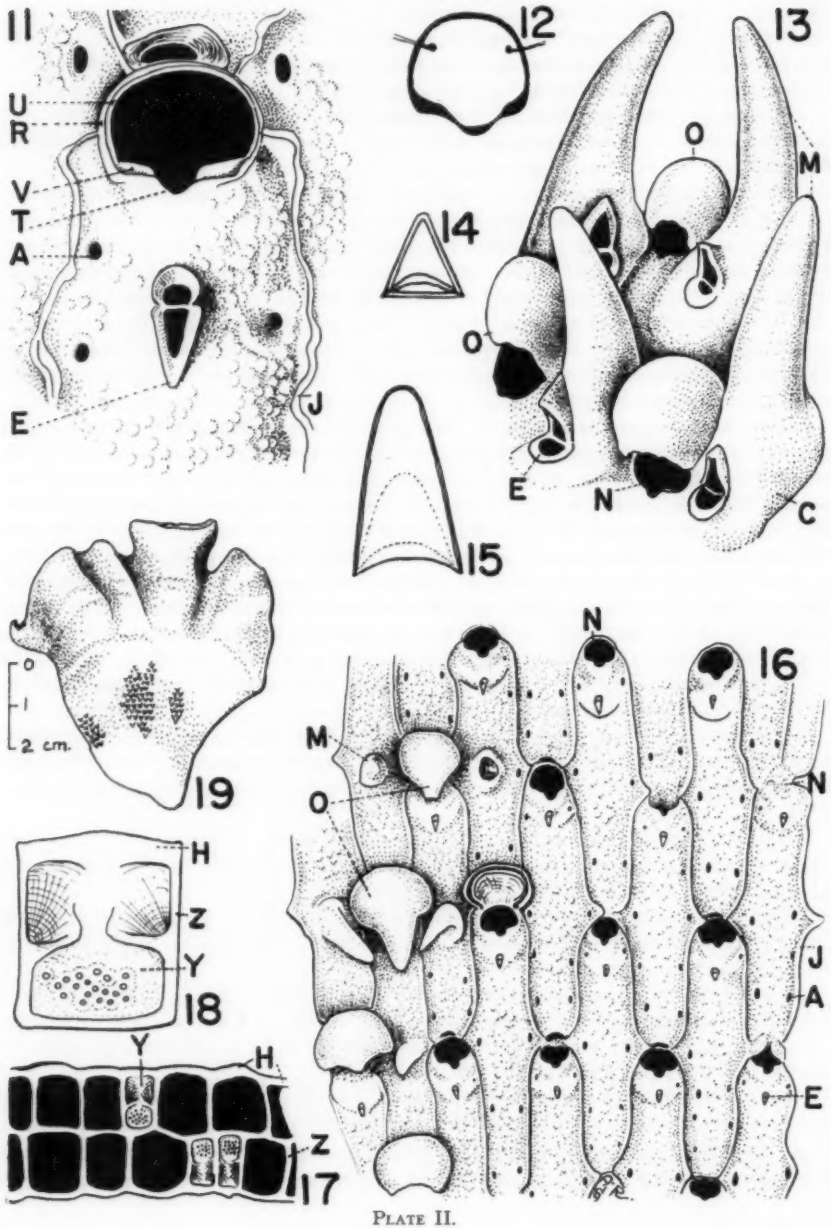

PLATE II.

front by a crease or groove that extends between the two paraorificial areolae (Fig. 5) but this later is obliterated. In *C. daedala* the cusps are transverse. In *T. absidatum* they are more vertical or obliquely arched.

Operculum. The operculum is rather delicate but reinforced by a long curved chitinous sclerite or reinforcement at each side (Figs. 7, 8). The sclerite is for muscle attachment. The extent or length of the sclerite depends on the degree of chitinization of the operculum. The operculum is shaped to fit the distal semicircular anter of the orifice and the intercardellar gap rather than the more external sinus.

Spines. Non-ovicelled zoids have 5 to 7 peristomial spines around the hemispherical part of the orifice. On ovicelled zoids the distal part of the orifice is not visible, so whether spine bases are present distally cannot at present be determined, but there is a thick spine at each side of the orifice just proximal to the side of the ovicell (Figs. 2, 5).

The spines are coarse and jointed at the thick base. The bases of the proximal pair are a bit sturdier or bigger on some zoids.

Ovicells. In general appearance the *Toretocheilum absidatum* ovicells resemble those of the genus *Stephanosella* and of Stach's *Chaestosella gabrieli* and Brown's *C. enigma.*

The *T. absidatum* ovicell is deeply immersed in the frontal of the next distal zoid. Also, it hides the distal part of the orifice of its own zoid. Its very own aperture cannot be seen from the front because the ovicell overhangs so. The peristome does not encroach upon the ovicell.

The ovicell has two calcareous layers, the ectooecium and the entooecium. Brown's (1952, p. 36) interpretation of these two layers is here followed. The layers are separated by a very narrow space. The entooecium is globose, rough-

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PLATE II

All figures on this plate are of *Isoschizoporella tricuspis* (Calvet) 1909. All except Figures 14 and 19 are drawn with a camera lucida. Figure 13 is from Sta. 226 material, the rest are from Sta. 234 specimens.

FIGURE 11. Detail of distal third of zooecium, showing well calcified beaded frontal wall and a characteristic uncalcified space above the orifice. The avicularium is prominent enough but the avicularial chamber is immersed and the heavy calcification makes it inconspicuous.

FIGURE 12. Operculum. Tendon fibers are attached to the two distal muscle dots.

FIGURE 13. Three ovicells flanked by aviculiferous spines.

FIGURE 14. Diagram of a suboral avicularial mandible.

FIGURE 15. Mandible of a spinal avicularium.

FIGURE 16. Portion of a colony showing both ovicelled and non-ovicelled zooecia. Some zooecia have the large frontal spines. One of the spines and one ovicell are broken, in the fourth row from the left. In the row at extreme right the two lower zooecia show degrees of occlusion of orifice by secondary calcification. The orifice of the upper one is completely calcified and overgrown. That of the lower right is partly so. The upper zooecium has given rise to two new rows of zooecia.

FIGURE 17. Cross-section through 15 zooecia of a bilaminate colony. Zooecial cavities are in black, zooecial walls are in white. The two layers of zooecia are back-to-back. The three filled in compartments are the end walls (*cf.* Fig. 18).

FIGURE 18. End or distal wall of a zooecium, showing the interzoidal communication pores or sieve plate in bottom half and the pattern or lines of calcification in the upper half.

FIGURE 19. Piece of a bilaminate blade or frond of a colony, drawn to the 2 cm. scale at left. The three darker patches on the lower half are spined and ovicelled areas.

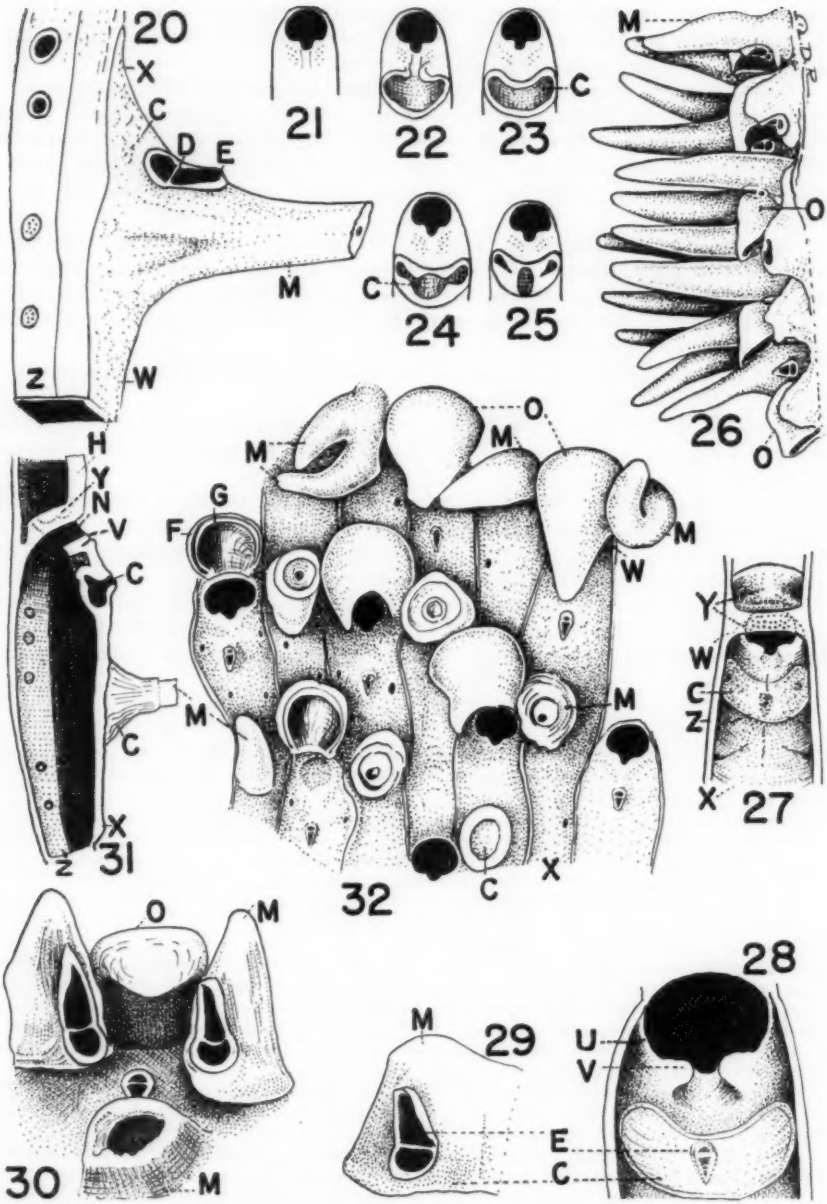
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PLATE III.

ened and complete. The ectooecium forms a partial incomplete shallow shell or band about the lateral and distal periphery of the entooecium.

Areolae occur in the ectooecium. Faint depressions emanate from these areolae. The ectooecium grows upward from around and between them to form the band about the entooecium. At the advancing border of the ectooecium the entooecial surface appears tucked or crowded because of the peculiar growth method or encroachment of the ectooecium. Not enough material was available for a more detailed study of the ovicell.

Distribution and ecology. A flat rock, measuring roughly about 17 cm. long, 10 cm. wide and 3 cm. thick, well encrusted with a dozen species of bryozoa and sponge, contained four small patches of *Toretocheilum absidatum.* These were on the side of the rock and colony fragments had to be chipped off or calcined off for study under the compound microscope.

The rock came from a depth of 40 fathoms, from USN Sta. 240 of Marguerite Bay, Antarctica, Comdr. D. C. Nutt collector. The specimens will be deposited

PLATE III

All figures on this plate are of *Isoschizoporella tricuspis.* All except Figures 21 through 25 are drawn with a camera lucida. Figures 20, 26, 29 and 32 are from Sta. 226 material. The rest are from Sta. 234.

FIGURE 20. Portion of zooecium, showing aviculiferous spine, frontal and lateral walls. The lateral wall has two pore plates (the two lower porous discs) and the "corresponding openings" (the two upper doughnut-shaped discs). The pore plates of one zooecium line up with the corresponding openings of its neighbor zooecia and vice versa.

FIGURES 21 THROUGH 25. Diagrams of external surface of frontal wall, depicting stages in the formation of the suboral avicularial chamber.

FIGURE 21. Zooecial front before an avicularial chamber has begun to form.

FIGURE 22. The avicularial chamber is outlined by a growing calcareous rim. The border is still incomplete distally.

FIGURE 23. Avicularial chamber now completely outlined.

FIGURE 24. Borders of the avicularial chamber growing and approximating.

FIGURE 25. Roofing over and fusion stage of avicularial chamber formation. Three open spaces still remain but these will be reduced in time to two areolar pores and an avicularium.

FIGURE 26. Side view of a spiny patch of colony with hooded ovicells between the spines. The spines at extreme right are either low or broken off and the ovicells can be seen just beyond them.

FIGURE 27. Back wall removed to show the interior of the front wall and the relative position of the distal wall. The distal wall with its sieve plate hides half the orifice. Cardelles and avicularial chamber also are visible from this side.

FIGURE 28. Enlargement of inner surface of orifice, cardelles, peristomial and vestibular sinuses and avicularial chamber. The avicularium shows through the translucent wall.

FIGURE 29. Early stage in the development of an aviculiferous spine. At this stage the avicularium and its chamber are present and the spine will result from the growth of the tip of the mound.

FIGURE 30. A more advanced stage in the growth of an aviculiferous spine. Two such spines flank a broken ovicell. Between their base lies a suboral avicularium. In the foreground is an exposed avicularial chamber of another aviculiferous spine (broken off).

FIGURE 31. Sagittal section through a zooecium. The darkest areas represent the zooecial and avicularial cavities. The upper three circles in the gray lateral wall are the pore plates. The two lower circles are the "corresponding openings" which would fit next to the pore plates of a neighbor zoid.

FIGURE 32. An ovicelled, spined, well calcified section of a colony. The upper tier of zooecia shows a curved spine and a double spine. The four spines of the middle and lower tiers are broken off. Ovicell shapes are variable. Two left ovicells are broken off and reveal the double calcareous wall.

with the Smithsonian Institution, U. S. National Museum, after the other species on the rock have been thoroughly studied.

GENUS *ISOSCHIZOPORELLA*, NEW GENUS

Diagnosis. Orifice suborbicular, with V-shaped sinus in its proximal lip. The two ends of the C-shaped vestibular arch stop at the sinus and form ledges (cardelles) to support the operculum. Operculum has two muscle dots distally and a proximal median tab to fit the sinus. Frontal wall smooth to beaded, flattened except for avicularial chamber and bordered by faint mural rim. Median suboral avicularium with mandible usually proximally directed present some distance below orifice. The reniform, bilaterally symmetrical avicularial chamber extends across the entire front wall and has a lateral pore on each side usually. Other avicularia are present in association with stout umbonate spines or tumid mounds. Frontal wall a pleurocyst, punctured by only a few peripheral pores spaced far apart and with bilateral symmetry. Ovicell globose to hood-like, smooth to granulated, imperforate, with double calcareous wall. Ovicells may grow downward over orifice.

Name derivation. The name *Isoschizoporella* was coined from the Greek word *isos* meaning like or equal and the already long existing generic name *Schizoporella* which was derived from *schizo* (to divide), *poros* (pore) and *ella* (diminutive).

Genotype. The genotype, here chosen for the new genus *Isoschizoporella,* is *Schizoporella tumida* var. *tricuspis* Calvet 1909, here elevated to species rank: *Isoschizoporella tricuspis* (Calvet).

Remarks. The reason for the erection of this new genus was two-fold. Firstly, there was need to set up a taxon for a troublesome species which had a schizoporellid orifice but which possessed other features which disqualified it from the presently restricted family Schizoporellidae. Secondly, the troublesome species seemed to belong to the family Hippoporinidae but possessed a totality of characters that would not permit its uncontested inclusion in any presently known hippoporinid genus. So it was thought best to erect a new genus for it, since this is a very rare form restricted to the Antarctic and sub-Antarctic regions.

The character by which *Isoschizoporella* differs from the family Schizoporellidae and resembles family Hippoporinidae is the nature of its frontal wall. *Isoschizoporella* has a pleurocyst, as does the family Hippoporinidae, while the Schizoporellidae have a tremocyst. A pleurocyst is a frontal wall that is granular, imperforate over the central area and perforated only around the periphery by areolar pores. A tremocyst is a frontal wall usually liberally sprinkled all over with pores.

The *Isoschizoporella* pleurocyst has a few elongate inconspicuous peripheral pores, very widely spaced, usually placed with bilateral symmetry at zooecial corners, and some elsewhere en route, like the paired pores of some Reteporidae as *Iodictyum* (Harmer, 1934; pp. 515, 522) in particular. The *Hippadenella carsonae* (Rogick, 1957b; Plate I) frontal is almost identical with that of *Isoschizoporella* but the orifice, operculum and ovicells are different.

Synonymy and previous records:

1909. *Schizoporella tumida* Hincks 1881, var. *tricuspis*. Calvet, pp. 28–30; Pl. III, Figs. 1–3. Excellent description and figures showing ovicelled and non-ovicelled zooecia, operculum and suboral avicularial mandible. No measurements given. From 30 meters' depth at Booth Wandel Isle and 110 meters' depth in Biscoe Bay.

1924. *Schizoporella tumida* var. *tricuspis*. Thornely, p. 12. Commonwealth Bay, Sta. 1, Lat. 66°50′ S., Long. 142°6′ E. at 354 fathoms.

1928. *Schizoporella tumida* var. *tricuspis*. Livingstone, pp. 7, 52.

1957a. *Schizoporella tumida* var. *tricuspis*. Rogick, p. 8, USN Sta. 226, Marguerite Bay. Ecological note.

The above are the only records of this species up to date.

Diagnosis. Colony bilaminate, foliaceous; smooth except for spiny or ovicelled patches. Zooecia long, narrow, flattened, bracket-shaped. Mural rims thin, faintly salient. Frontal a granulated or beaded pleurocyst with a few paired peripheral pores. A small median oval to pointed suboral avicularium sits atop the wide immersed reniform avicularial chamber. A stout tusklike aviculiferous process ("spine") occurs on the midfrontal of zooecia adjacent to an ovicelled zoid. Some zoids without spines or ovicells. Ovicell imperforate, granular, shaped like an elephant's head, overhanging the zooecial orifice, usually flanked on each side by its neighbors' aviculiferous frontal spines. Zooecial orifice and operculum as in genus. The median V-shaped peristomial sinus is less than half the width of the proximal lip. Zooecial orifice is at distal end of frontal and is touched by the frontals of the three adjacent zoids. Distal to orifice, at the beginning of the next zoid is a membranous area, crescent-shaped. Zooecial end wall a sieve plate. Four or five multiporous pore plates or corresponding openings present in each lateral wall.

Measurements. All readings are in millimeters. H is for height; D is for diameter at base.

1.181–1.991	(1.506)	L	zooecia, 20 readings
0.272–0.432	(0.351)	W	zooecia, 20 readings
0.144–0.173	(0.158)	L	orifice, including sinus
0.173–0.202	(0.184)	W	orifice
0.143–0.176	(0.163)	L	operculum
0.166–0.195	(0.182)	W	operculum
0.446–0.619	(0.531)	L	ovicell, including its beak
0.317–0.461	(0.413)	W	ovicell
0.072–0.115	(0.093)	L	suboral avicularium
0.043–0.072	(0.055)	W	suboral avicularium
0.156–0.234	(0.190)	L	spinal avicularium, 8 readings
0.091–0.137	(0.107)	W	spinal avicularium, 8 readings
0.760–1.177	(1.039)	H	frontal spine
0.377–0.514	(0.456)	D	at widest part of spine base avicularial chamber
0.260–0.358	(0.311)	D	at narrowest part of spine base avicularial chamber
0.046–0.078	(0.061)	L	mandible of suboral avicularium

0.039–0.065 (0.051) W mandible of suboral avicularium
0.117–0.130 (0.124) L mandible of spinal avicularium, 2 readings
(0.130) W mandible of spinal avicularium, 1 reading

Remarks. Calvet's hesitancy (1909, p. 28) about the identity of his *tricuspis* and its exact relationship to *Schizoporella tumida* Hincks (1881, p. 13, Pl. I, Fig. 3) is understandable in view of the brevity and incompleteness of Hincks' description and illustration. Hincks' figure shows four non-ovicelled zooecia and a mound bearing a special avicularium. This mound is described by Hincks as "frequently an ovate rising on the side of the cell extending from the orifice down a considerable portion of its length, bearing on its upper extremity an immersed avicularium, with pointed mandible directed downwards." The *S. tumida* ovicell is described as "globose and prominent, with a smooth surface."

Hincks neither mentions nor figures the frontal pores or frontal spines and the ovicell is apparently without the downward orifice-covering beak. Also, the position of the suboral avicularium is much closer to the orifice in *S. tumida* than in *Isoschizoporella tricuspis*. *Schizoporella tumida* Hincks was collected in the Bass Straits of Australia, while *Isoschizoporella tricuspis* is known only from the Antarctic. In these ways then does *S. tumida* differ from Calvet's var. *tricuspis*, so it is best to elevate Calvet's variety to species rank.

Colony. Colony color is light écru. Judging from the fragments the colony should be good sized, foliaceous or fan-shaped, with crinkled edges. In the USN collection were a number of chips or fragments, none of them a complete colony. The largest piece was 67 mm. long by 60 mm. wide, and probably represented less than half a colony because it was a portion of the growing edge, apparently some distance away from the base of the colony, judging by its width.

Some of the pieces have occasional faint markings, like growth rings (Fig. 19). New linear or radial rows of zooecia are added in such zones, or when needed, to make possible the ever widening periphery or ruffled edge of the colony.

Colonies are bilaminate and solid, with zooecia back to back, and mostly smooth. Occasional bristly patches occur on either face (Figs. 13, 16, 19, 26). These patches represent areas where ovicells and aviculiferous spines have arisen. One such patch was 12 mm. long by 10 mm. wide.

Sometimes secondary calcification closes over the zooecial orifices (Fig. 16) but the suboral avicularia and frontal pores seem much more resistant to secondary filling-in. The secondary calcification seems to begin with the operculum itself. It fills in or frosts over lightly, then more solidly till the whole orifice is like the surrounding frontals.

Colonies are relatively clean, living zoids apparently being resistant to encrustation with extraneous material, but dead parts show some settlers.

Soft parts. Many zoids had opercula and mandibles. Far fewer contained polypides (gut, tentacles, musculature). These polypide remains, when present, were very slim, in keeping with the slenderness and elongation of the zooecia and also perhaps the food supply was insufficient. Some of the zoids had the opercula fouled or rimmed about by a border of scummy orange-colored material, as if wastes had accumulated about the orifice and killed the zooecia. It was not possible to determine if embryos were present in ovicells because the ovicells are so opaque.

Suboral avicularium. A suboral avicularium develops on nearly every zoid. It is median, oval, with a triangular mandible that is proximally directed (*cf.* Figs. 11, 14). Most of the suboral avicularia are so oriented longitudinally but occasional ones do occur a bit off course. That is, their slant may be slightly oblique, to right or left. One colony fragment from USN Sta. 234 contained a zoid whose suboral avicularium pointed transversely while the rest of the suboral avicularia in that fragment were oriented in the usual manner.

Sometimes the mandibular area tip (rostrum) is flat against the frontal ("chest"), other times its tip is more elevated.

The suboral avicularium forms after its reniform chamber is complete (Figs. 11, 21–25, 27, 28, 31). The floor of the chamber is the zooecial frontal wall. The chamber stretches from lateral wall to lateral wall. The chamber walls grow upward from the zooecial frontal, converge in a transverse line that has three diminishing gaps in it (Figs. 22–25). These gaps are the two lateral pores and the median avicularium. The mandibular area, back area and pivot develop over the median gap. At the base of the chamber, particularly on the lateral side, can be found the aforementioned pore and perhaps another one, puncturing the chamber.

Aviculiferous spines. The big tusk-like frontal spines are present on some zoids, either ovicelled or non-ovicelled, and absent from others. They are aviculiferous, *i.e.,* house an avicularium and avicularial chamber in their base (Figs. 13, 20, 30, 31). When present, there is generally only one spine per zoid. An occasional spine may be forked (Fig. 32) but most of them are not. Some of the spines are straight, some curved. They are tall, thick-walled, wide at the base and taper to a blunt tip and sometimes look corrugated. They are hollow, the basal part being especially large (Figs. 20, 31). The cavity of the spine is not continuous with the cavity of the zooecium. The basal part of the spine cavity is the avicularial chamber.

These spines arise as calcareous blisters on the midfrontal wall of a zoid (Figs. 31, 32). They sprout close to a neighbor's orifice and ovicell as if to protect both (Figs. 16, 32). One wonders what influence, if any, the developing ovicell or developing embryo of a zoid exerts on neighboring zoids to cause them to produce the protective aviculiferous spines.

When still in the first stage of formation the avicularial spine base is very extensive. One was 0.247 mm. wide by 0.52 mm. long, as wide as some of the zooecial fronts. The base is oval or reniform in outline. Strictly speaking the spine base represents the avicularial chamber.

The avicularium at first is oblique in position like a door leaning against a mound. But, with the continued upward growth of the mound to form a hollow spine the position of the avicularium shifts until it is roughly vertical, *i.e.,* at right angles to the zooecial plane. The avicularium appears as if propped up against or incorporated into the proximo-lateral side of the spine. Its duckbill-shaped mandible (Fig. 15) is larger than the triangular mandible of the suboral avicularium. Also, the suboral avicularium itself is smaller than the spine avicularium. The mandible of the spine avicularium is directed toward the tip of the spine (Figs. 20, 26).

In the USN material these aviculiferous spines develop in the vicinity of ovicells or orifices of ovicelligerous zoids, leaning toward the ovicells. Generally an ovicell

has an aviculiferous spine on each side of it (Figs. 13, 16) but there can be exceptions. Occasional ovicells may share a spine between them. Still others may have but a single spine beside them.

Interzoidal communications. In *I. tricuspis* the end walls of zooecia are generally single partitions or transverse septa between succeeding zoids in a linear or radial series, *i.e.*, two zoids share the same single end wall between them. The lateral walls are double, each neighboring zoid having its own individual lateral wall. Therefore it is not surprising that the interzoidal communications between zoids should be different in the two different kinds of walls. Silén (1944) made an extensive study of this situation in a number of different species.

In *I. tricuspis* the transverse and the lateral walls are perforated, but differently. The end wall is truncated, *i.e.*, divided into two sections, one with the porous sieve plate, the other without pores (Figs. 17, 18, 31). The sieve plate is nearer the basal wall than is the non-porous section and slants away from the basal wall at about a 45° angle. The other section is more nearly vertical with respect to the basal wall. The number of closely set pores in the slanting sieve plate is hard to count but one zoid had about 16, more or less.

The lateral wall has 4 or 5 multiporous pore chambers or corresponding openings (Figs. 20, 31). These blister-like pore chambers have fewer pores, about 3 to 8, than do the sieve plates.

Distribution and ecology. A small number of fragments, about 18, and none of them a complete colony, of *Isoschizoporella tricuspis,* were collected by the 1947–1948 USN Expedition from two stations in Marguerite Bay, Antarctica, Sta. 226 and 234, from depths of 40 fathoms. These localities are near that from which Calvet's specimens came. The Thornely specimens were collected at opposite sides of the Antarctic continent and from a considerably greater depth, 354 fathoms. So, although the species showed up in very small quantity in the collections of only three Antarctic expeditions, its occurrence at opposite sides of the pole would suggest a circumpolar distribution.

The USN specimens were relatively clean of encrustation by other forms but a few bryozoans did gain a foothold on some of the fragments. One *Beania erecta* zoid and a lichenoporoid cyclostomatous bryozoan colony, to be identified later, grew on the Sta. 226 fragment. Species found on the Sta. 234 fragments included the bryozoans *Osthimosia milleporoides* (Calvet) 1909, *Phylactellipora lyrulata* (Calvet) 1909 and *Ramphonotus inermis* (Kluge) 1914 and foraminifera.

The *I. tricuspis* specimens will be deposited with the Smithsonian Institution, U. S. National Museum.

Summary

1. Two new genera, *Toretocheilum* and *Isoschizoporella,* of the family Hippoporinidae (Bryozoa, Cheilostomata) have been erected on the basis of their orifices and areolated frontal walls.

2. One new species, *Toretocheilum absidatum,* was described. Another, *Schizoporella tumida* var. *tricuspis* Calvet 1909, was elevated to species rank and transferred to a new genus: *Isoschizoporella tricuspis.*

3. Both species are amply illustrated, measurements given and the description for *I. tricuspis* has been amplified. Attention is given to the range of variation of each species.

4. Each species is designated as the genotype of its new genus. Both species are from Marguerite Bay, Antarctica.

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DISCONTINUOUS RESPIRATION IN INSECTS: ROLE OF THE SPIRACLES [1]

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In many groups of insects [2] metabolic carbon dioxide is retained within the insect and released during brief periods in "bursts" (Punt, 1944, 1948, 1950a, 1950b, 1956; Schneiderman, 1953; Schneiderman and Williams, 1953–1955; Buck *et al.*, 1953; Buck and Keister, 1955; Ito, 1954). In diapausing pupae of the Cecropia silkworm, for example, more than nine-tenths of the metabolic carbon dioxide is stored and then released in brief bursts, which occur from once every week to many times per hour, depending on the temperature and metabolic rate. The remaining carbon dioxide escapes during the interburst period. When measured by usual manometric procedures, the uptake of oxygen, unlike the release of carbon dioxide, appears continuous and almost steady (Schneiderman and Williams, 1953a, 1955; Buck and Keister, 1954, 1955). If the spiracles are sealed with paraffin, virtually all the respiratory exchange ceases (Ito, 1953; Schneiderman and Williams, 1955); the spiracles are, therefore, the site of both the discontinuous release of carbon dioxide and the simultaneous continuous uptake of oxygen. When metabolic rate is low, the bursts are accentuated. Thus at 10° C. carbon dioxide may be given off only once a week and the interburst rate of carbon dioxide may be but 1/100th the rate of oxygen uptake. This indicates the true dimensions of the respiratory paradox: oxygen enters the spiracles during the interburst period at many times the rate at which carbon dioxide leaves and, furthermore, the insect releases its carbon dioxide periodically.

The central importance of spiracular behavior in the discontinuous release of carbon dioxide was suggested by the observation of Buck and his co-workers (1953, 1955) that intubating the spiracles of *Agapema* pupae eliminated the bursts of carbon dioxide. Subsequently, we observed that excision of the valve from one of the fourteen functional spiracles of a Cecropia pupa caused carbon dioxide output to become continuous (Schneiderman and Beckel, 1954; Schneiderman, 1956). The only maneuver that restored the discontinuous release of carbon dioxide was the sealing of the open spiracle. It appears from this experiment that all the spiracles

[1] This research was aided by a research grant, H-1887, from the National Heart Institute of the United States Public Health Service.

[2] Groups include larval and adult Hemiptera (*Rhodnius prolixus; Triatoma rubrofasciata, Cimex lectularius;* adult Dictyoptera (*Periplaneta americana*); larval and adult Orthoptera (*Locusta migratoria*); adult Coleoptera (*Carabus nemoralis, Meloë proscarabaeus, Hadrocarabus problematicus*); diapausing larvae of Lepidoptera (*Arctia* sp.); diapausing pupae of Lepidoptera (*Hyalophora cecropia, Antheraea polyphemus, Samia cynthia, Rothschildia oryzaba, Agapema galbina, Bombyx mori* ("dauer pupae"), *Sphinx ligustri, Agrotis* sp., *Papilio macheon*); non-diapausing pupae of *Bombyx mori*, diapausing adults of Lepidoptera (*Vanessa urticae*).

remain nearly closed during the interburst, preventing carbon dioxide from diffusing out in substantial quantities, and that one or more of the spiracles opens during a burst. The spiracular valves thus hold an important key to the discontinuous release of carbon dioxide.

Several theories have been proposed to explain the disparate rates of oxygen and carbon dioxide transfer during the interburst period, as well as the bursts themselves (Punt, 1944, 1950a; Punt *et al.*, 1957; Zeuthen, 1955; Buck *et al.*, 1953; Buck and Keister, 1954, 1955; Schneiderman, 1956). The most recent and comprehensive theoretical analysis of the entire phenomenon is that of Buck (1958a, 1958b). This theory and all of the others depended for the most part upon (a) indirect estimations of tracheal carbon dioxide and oxygen tensions during the "burst cycle," (b) postulated behavior of the valves that regulate the opening of the spiracles, (c) assumed changes in intratracheal barometric pressure for which there was no empirical evidence whatever, (d) hypothetical changes in the volume of the tracheal system, (e) cataclysmic biochemical changes. To test these theories and to resolve the paradox, it proved necessary: (a) to continuously record the behavior of the spiracular valves during the burst cycle, (b) to measure directly the changing composition of tracheal gases during the cycle, (c) to measure the changing intratracheal barometric pressure, and (d) the changing tracheal volume during the cycle. The present report initiates a series in which methods will be described that accomplish these objectives and provide fairly precise pictures of both the partial and absolute pressure gradients driving oxygen into the insect, the gradients driving carbon dioxide out of the insect, and cyclic variations in the aperture of the spiracles and in the volume of the tracheal system. The results to be reported confirm that the breathing of silkworm pupae involves processes other than physical diffusion, and also bear out many of the theoretical predictions of Buck (1958b). However, in some essential points, they do not support his theory, but instead provide evidence for another kind of insect breathing which seems different from any previously proposed or demonstrated and which may account for discontinuous respiration. In addition, they define the stimuli that cause the cyclical activity of the spiracles.

The first paper focusses on the role of the spiracles in discontinuous respiration. A preliminary account of some of these results has been given elsewhere (Schneiderman and Beckel, 1954; Schneiderman, 1956).

Materials and Methods

1. *Experimental animals*

Experiments were performed on diapausing pupae and developing adults of the giant Saturniid silkworms *Hyalophora cecropia, Antheraea polyphemus* and *Samia cynthia.* In our experience these species of closely related moths behave in virtually identical fashion in the sorts of experiments that were undertaken. Animals were reared on net-covered trees or purchased from dealers and stored at 25° C. and 60% to 70% relative humidity. All experiments were conducted at 25° ± 1° C.

2. *Respiratory measurements*

Respiratory exchange was determined manometrically by techniques previously described (Schneiderman and Williams, 1953a, 1955). Measurements were per-

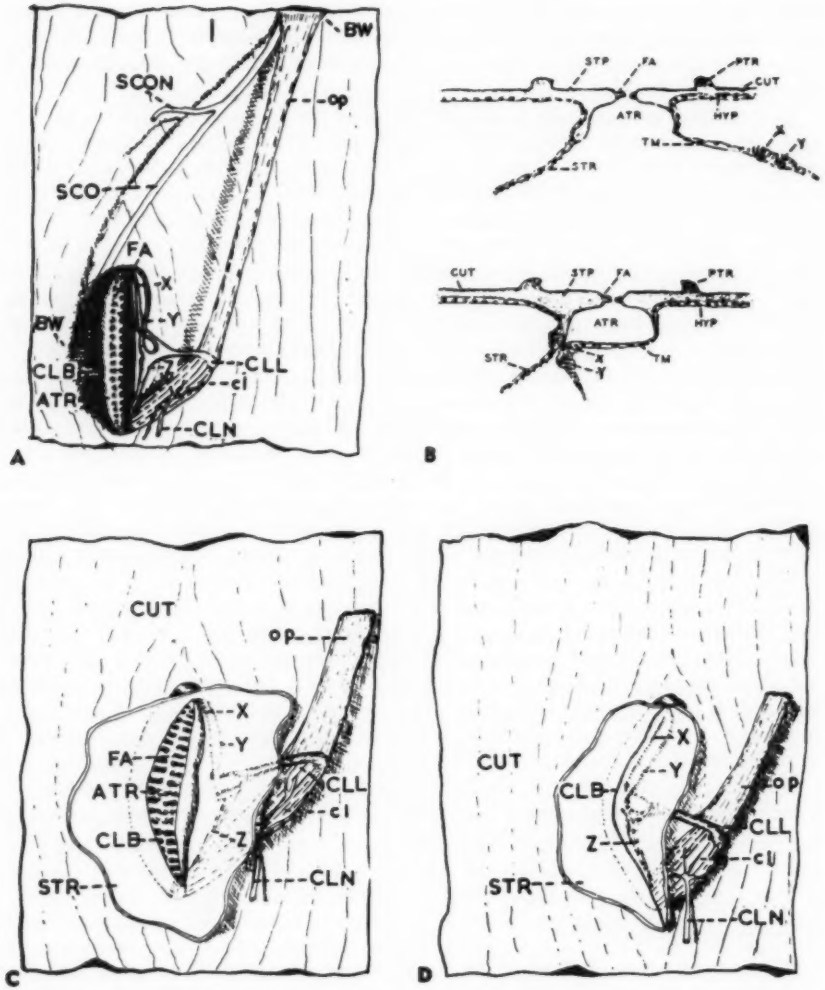


FIGURE 1. (A) Pupal spiracular regulatory apparatus of Cecropia, as seen from inside the animal. No tracheae are shown. *ATR*, atrium; *BW*, body wall; *cl*, closer muscle; *CLB*, closing bow; *CLL*, closing lever; *CLN*, nerve of closer muscle; *FA*, filter apparatus or stigmal plate; *OP*, elastic opener; *SCO*, scolopophorous organ; *SCON*, nerve of scolopophorous organ; *X*, dorsolateral closing bar; *Y*, dorsomedian closing bar; *Z*, ventral closing bar. (B) Frontal sections through the spiracular region, above the closing lever, and cutting the two dorsal closing bars. These show how the spiracle closes when the closing bars are pushed against the closing bow or atrium. *CUT*, cuticle; *HYP*, hypodermis; *PTR*, peritreme; *STP*, stigmal plate, *STR*, spiracular tracheal manifold; *TM*, tracheal membrane. (C) Apparatus viewed from the inside with the scolopophorous organ and part of the elastic opener removed. Only part of the spiracular tracheal manifold is shown. The valve is open. (D) Same as (C) only the valve is closed.

formed by the "direct manometric method" (Umbreit *et al.*, 1958) in 45-cc. cylindrical vessels equipped with venting plugs and adapters, for use with standard Warburg manometers.

3. *Recording of spiracular movement*

(a) *Anatomy of the spiracle*

These silkworm pupae have a pair of thoracic spiracles and six pairs of functional abdominal spiracles. Each spiracle is surrounded by a chitinous peritreme, and covered by a stigmal plate, or filter apparatus, which communicates by a thin slit to a chamber below—the atrium—which contains the spiracular valve. Gas exchange between the atrial chamber (and hence the atmosphere) and the interconnecting tracheal system, which lies just below it, is regulated by this valve. The morphology of the spiracular apparatus and some of the physiological properties of the spiracular muscle of pupae of Saturniid moths have been described most recently by Beckel (Beckel, 1955, 1958; Beckel and Schneiderman, 1956, 1957). For our present purposes, it is sufficient to note that the spiracular valve is an epithelial membrane which is firmly attached to a chitinous frame consisting of a bow and three bars which unite in the middle to give rise to a lever (Fig. 1). A closer-muscle stretches from the ventral tip of the lever to the ventral corner of the valve. It is opposed by an elastic ligament which extends from the dorsal tip of the lever to the body wall. When the closer muscle contracts, it pulls on the lever and closes the valve. When it relaxes, the valve opens because of the elasticity of the chitinous frame and the tension of the opposing elastic ligament. The muscle is innervated by a nerve from the corresponding segmental ganglion and by a branch of the median nerve of the next anterior segment.

(b) *Recording valve movements*

To expose the valves in the living insect it was necessary to remove the overlying chitinous stigmal plate. This could easily be done under the dissecting microscope after the animal had been anaesthetized with carbon dioxide. Insofar as could be judged, this operation in no way interfered with the normal functioning of the valves, which were now clearly visible. When the valves were so exposed for a week or more, they occasionally dried out and ceased functioning normally. To prevent this, after each period of observation, the spiracular opening was sealed with Tackiwax.[3] Because of their greater accessibility, the abdominal spiracles were examined in preference to the thoracic pair. Of these the first, second, and third abdominal were the easiest to study because they served non-collapsible segments and were not obscured when the animal moved its abdomen.

Several methods for recording spiracular movements of intact insects have been employed in the past and there have been several descriptions of spiracular behavior in the cockroach *Periplaneta americana* (Hazelhoff, 1926a, 1926b), the phasmid *Dixippus morosus* (Stahn, 1929); the rat flea *Xenopsylla cheopis* (Wigglesworth, 1935; Herford, 1938), the bedbug *Cimex lectularius* (Wigglesworth, 1941), the

[3] After the present experiments were completed, we discovered that a valve can be prevented from drying out by sealing a small transparent plastic window over the exposed spiracle. Although the window prevents gas exchange through the spiracle, it allows convenient observation of spiracular movements, and the valve functions for many weeks.

grasshoppers *Dissosteira carolina* (McCutcheon, 1940), *Schistocerca obscura* (Watts, 1951), and *Schistocerca gregaria* (Hoyle, 1959), the larva of the commercial silkworm (Shimizu and Ono, 1942), the flies *Musca domestica* and *Callitroga macelleria* (Case, 1956a) and the cockroaches *P. americana* and *Blaberus craniifer* (Case, 1957b). The older literature has been summarized by Hazelhoff (1926a). The present experiments employed a modification of the ocular micrometer system of McCutcheon. A pupa with one or more of its spiracles exposed to view was placed beneath a binocular dissecting microscope furnished with an ocular micrometer and examined under 60 ×. The hairline of the micrometer was focussed on the leading edge of the spiracular valve and was adjusted to follow the valve as it opened and closed (Fig. 2). The crosshairs on the micrometer eyepiece indicated the position of the hairline when the valve was closed. The hairline was moved by a rotating knob whose action was translated into a kymograph trace by means of a system of pulleys and levers and an ink-writing pen recording on a

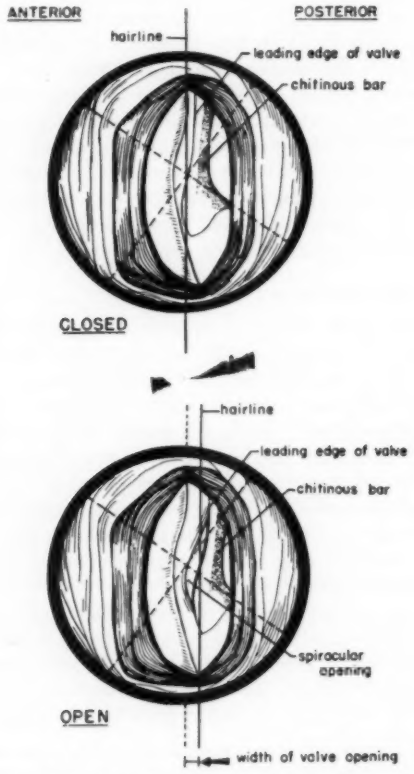


FIGURE 2. Spiracle as seen through ocular micrometer. The filter apparatus and most of the stigmal plate have been removed and the valve is visible. For further details see text.

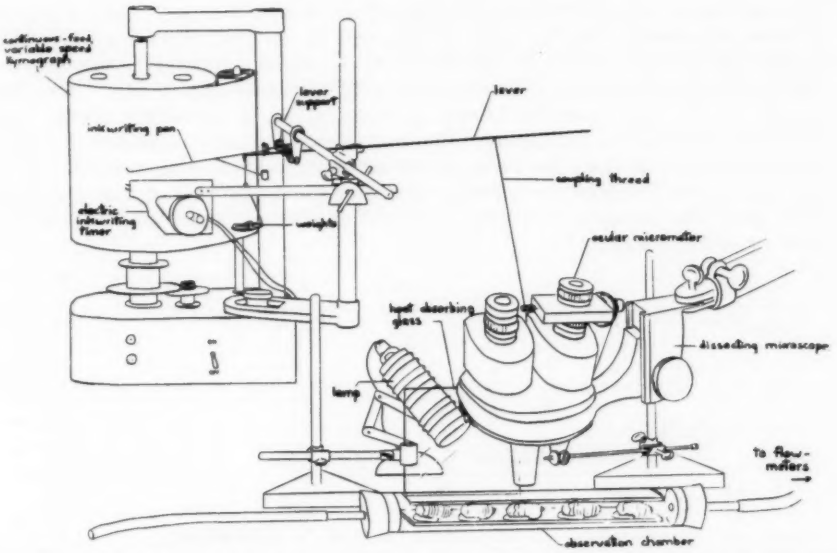


FIGURE 3. Apparatus used for recording spiracular valve movements. The observation chamber shown is of the sort also employed in studies with intubated pupae. For further details see text.

continuous feed kymograph (Fig. 3). The tracings of the ink-writing pen provided a record of the movements of the spiracular valve, each deflection on the record representing a valve movement. The ordinate of the trace shows the width of valve opening in arbitrary units, while the abscissa denotes the duration of the opening. Directly beneath the record of valve movements the time was recorded by an ink-writing timer.

In most of the experiments spiracular behavior was viewed through a 20-cc. glass chamber in which the insect was held secure in a plasticene support, or in a 250-cc. lucite chamber with a flat surface for optical convenience. Air and gas mixtures were flushed through the chamber as desired. The rate of gas flow varied from 25 to about 500 cc./min. in different experiments and appeared to have no effect on the phenomena under investigation. The time required to change the atmosphere in the small chamber was rarely longer than 10 seconds, but for the large chamber it took about two minutes.

Gas mixtures were either pre-mixed in pressure cylinders or made up by proportional flow. All mixtures were analyzed periodically.

A. EXPERIMENTS WITH INTACT PUPAE

1. *Normal behavior of spiracles*

Figure 4 is a portion of a typical record of the activity of the third abdominal spiracle of a Cecropia pupa over a period of four hours. A consistent pattern of

valve movement occurred in cycles lasting 45 minutes to 2 hours. After a period of about 10 minutes, during which it remained closed and motionless, the valve fluttered for between 15 and 40 minutes and then, within a minute, the valve movements progressively increased in amplitude until the valve opened fully. Swaying slightly, it remained open for several minutes. Then it alternately opened and closed for several minutes, and the valve movements gradually decreased in amplitude and duration, until the valve closed altogether. Following this, the valve remained motionless until the fluttering preceding the next period of wide openings. This cycle was repeated over and over, and apparently represented the normal behavior of the spiracle.

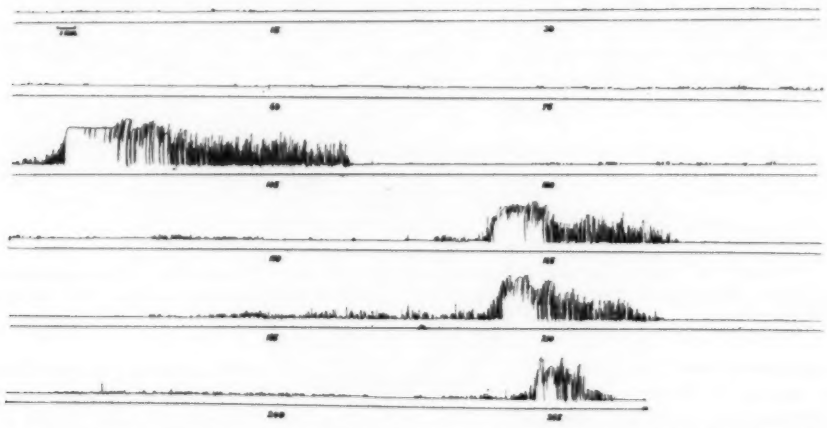


FIGURE 4. Record of the valve movements of a third abdominal spiracle of a diapausing Cecropia pupa over a 4-hour period in air at 25° C. Each mark above the baseline represents a valve opening. Occasional marks below the baseline are artifacts caused by vibration of the apparatus.

Manometric observations of the carbon dioxide output of this pupa revealed bursts of carbon dioxide at intervals of 30 minutes to two hours. A similar correlation between the frequency of carbon dioxide bursts and periods when the valve was widely open has been consistently observed in all other silkworm pupae studied. Moreover, by utilizing techniques which permit simultaneous recording of both valve movements and gas exchange, it has been possible to show the correlation directly (Schechter and Schneiderman, unpublished observations). Hence it seems evident that the periodic bursts of carbon dioxide result from the periodical prolonged openings of the spiracles, which we have termed "spiracular bursts." The term "burst" seems appropriate for both the spiracular and the manometric events, since they coincide. The spiracular burst can conveniently be partitioned into a period of wide openings—the "open phase"—which is followed by a period of rapid closures—the "decline phase." The end of the decline period and of the spiracular burst is marked by the moment the valve constricts tightly. The "interburst"

consists of a "constriction period" after the burst, when the valve appears closed and motionless, and a "flutter period" prior to the next burst.

The flutter period was usually quite irregular in terms of both the frequency and amplitude of the valve movements. As Figure 4 reveals, the flutters usually

TABLE I

Duration of various phases of spiracular burst cycles in a typical series of diapausing Cecropia pupae and developing adults of Polyphemus

Experiment No.***	Cycle length (minutes)	Duration of various phases (minutes)*			
		Interburst		Spiracular burst	
		Constriction	Flutter	Open	Decline
Cecropia					
827	114	13 (11)	91.5 (80)	5.5 (4.8)	4.0 (3.5)
8316	74	10 (14)	61 (81)	1.0 (1.4)	2.0 (2.7)
938	73	26 (36)	42 (57)	1.5 (2.1)	3.5 (4.8)
739	68	26 (38)	37.5 (55)	1.0 (1.5)	3.5 (5.1)
8315	65	12 (19)	49 (75)	1.0 (1.6)	3.0 (4.7)
625 a	63	10 (16)	43 (68)	3.0 (4.8)	7.0 (11.1)
8314	50	16 (32)	29.5 (59)	1.5 (3.0)	3.0 (6.0)
625 b	44	16 (36)	19 (43)	2.5 (5.7)	6.5 (14.8)
725 c	43	12 (28)	26 (61)	2.0 (4.7)	3.0 (6.9)
8311 b	39	12.5 (32)	23.5 (60)	1.0 (2.6)	2.0 (5.1)
439	38	17 (45)	16 (42)	1.0 (2.6)	4.0 (10.5)
8311 c	32	18 (56)	12.5 (39)	1.0 (3.1)	0.5 (1.6)
8311 a	29.5	7.5 (25)	19 (65)	1.5 (5.1)	1.5 (5.1)
Average**	56.35 ± 23.25	15.08 ± 5.71	36.12 ± 21.96	1.81 ± 1.28	3.35 ± 1.82
Average %	100	29.85 ± 12.94	60.38 ± 13.67	3.31 ± 1.52	6.30 ± 3.71
Polyphemus					
522 a	16.5	0	15.0 (91)	—	—
522 b	15.4	0	12.3 (80)	—	—
527 a	10.7	0	6.4 (60)	—	—
527 c	9.7	0	7.0 (72)	—	—
527 d	9.1	0	6.2 (68)	—	—
527 f	8.1	0	5.5 (68)	—	—
5218	7.0	0	4.6 (66)	—	—
113 a	6.0	0	3.5 (58)	1.5 (25)	1.0 (17)
113 b	6.0	0	3.0 (50)	2.0 (33)	1.0 (17)
114	6.0	0	3.6 (60)	1.7 (28)	0.7 (12)
115	5.5	0	2.5 (45)	2.3 (42)	0.7 (13)
521 c	5.5	0	3.5 (64)	—	—
117	4.3	0	2.8 (65)	1.0 (23)	0.5 (12)
521 c	2.7	0	1.4 (52)	—	—
Average	8.04 ± 3.98	0	5.52 ± 3.84	1.70	0.78
Average %	100	0	64.21 ± 11.94	30.2	14.2

* Figures in parentheses record the average per cent of the cycle length.

** ± standard deviation.

*** In this and subsequent experiments, the first two digits refer to the particular animal employed, *i.e.*, all experiments beginning with "82" refer to pupa No. 82.

came in volleys which lasted from 10 seconds to 8 minutes and which were punctuated by closed periods which lasted from 20 seconds to several minutes. Less commonly, flutters occurred singly. Frequency varied from short volleys of about one per second to long volleys of one opening every two to ten seconds. The openings were invariably brief, commonly lasting less than a second. Amplitude also varied somewhat and an occasional wide opening punctuated a series of smaller ones. There appeared to be no systematic variation in amplitude, however, until a minute or two before the burst.

Several hundred hours of records of such cycles have been obtained from about 30 individual pupae. Table I records the duration of the various phases of several typical cycles recorded from diapausing Cecropia pupae and developing adults of Polyphemus. Although the cycles that were studied varied in frequency from 20 per hour to one every two hours, the pattern of valve movements in all cases was remarkably similar. The only variation occurred in pupae with cycles of 15 minutes or less. Here the constriction period was lacking and the spiracular valve was in constant slight motion (see Figures 6 and 7). Also, in pupae with brief cycles the frequency of flutters was considerably greater than in pupae with long cycles, and the spiracular bursts occupied a larger and larger proportion of the cycle.

Simultaneous observations of two or three spiracles on the same side indicated that the spiracles were coordinated. When the valves opened in a burst or closed at the end of a burst, they did so within a minute of each other, though when they fluttered, the pulsations were not in exact synchrony. For our present purposes, these observations indicate that recording the behavior of one spiracle provides an accurate picture of the behavior of all the spiracles of the animal, except possibly the thoracic spiracles whose behavior we have never succeeded in observing.

Previous experiments (Punt, 1950a; Schneiderman and Williams, 1955; Buck and Keister, 1955) pointed out that various factors such as metabolic rate, oxygen and carbon dioxide tensions, etc., profoundly affected the cyclical release of carbon

TABLE II

Burst cycle of a Polyphemus during adult development

Days of adult development	Cycle length (minutes)	Spiracular burst duration (minutes)	Amount of interburst fluttering*
−13	120.0	6.8	+
0	9.8	3.3	++
2	7.0	2.4	++
3	5.0	2.7	++
4	5.4	2.0	++
5	4.4	1.7	++
6	4.7	2.0	+++
9	4.0	1.7	+++
10	4.3	2.0	+++
15	2.7	1.3	++++
16	0	0	++++
20 (emergence)	0	0	++++

* +: Normal amplitude, moderate frequency; ++: normal amplitude, high frequency; +++: greater than normal amplitude, high frequency; ++++: fluttering about half-open position, high frequency.

dioxide. Recognizing the key role of the spiracular valves, it was reasonable to anticipate that these several factors would influence the movement of the spiracular valves. This is clearly shown in the following experiments.

2. Effects of metabolic rate on the behavior of the spiracular valves

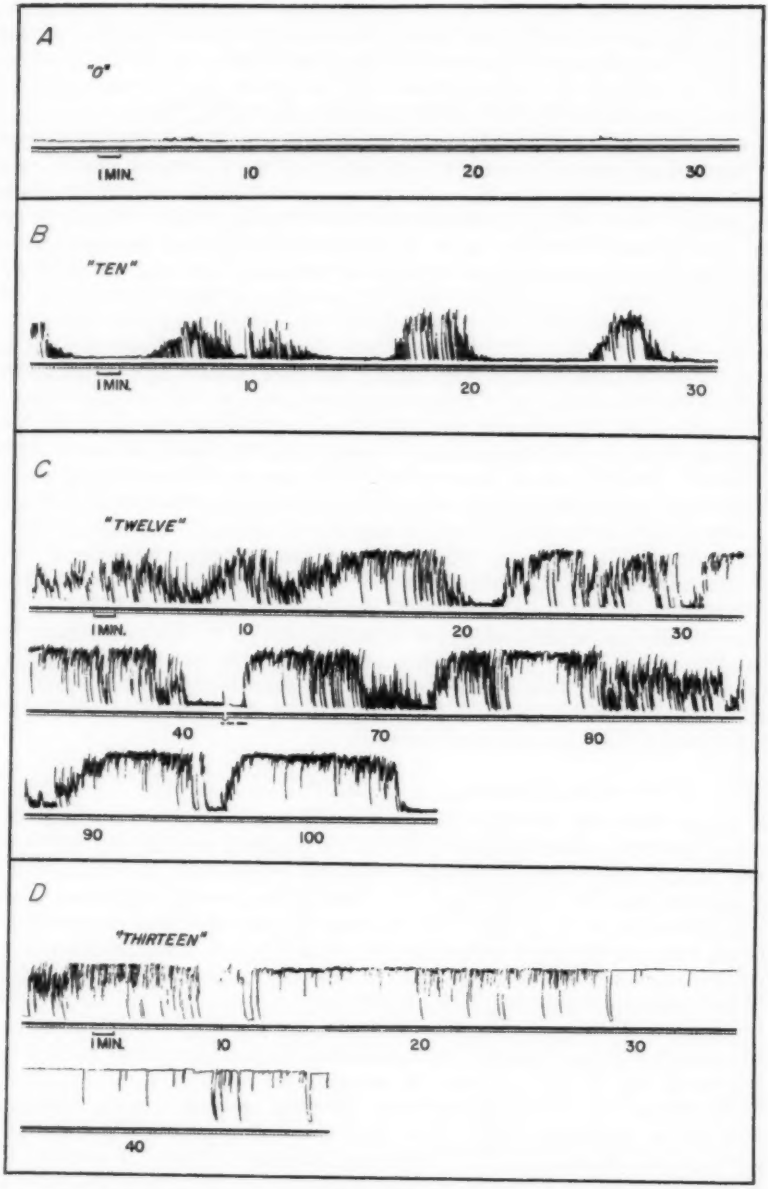
During the pupal-adult transformation, oxygen uptake rises markedly, the cycles of carbon dioxide release become more frequent and the interburst rate of carbon dioxide output increases until the cycles disappear and carbon dioxide is released continuously (Punt, 1950a; Schneiderman and Williams, 1955). Table II summarizes the spiracular behavior of a Polyphemus during adult development, and reveals that the spiracular bursts increased in frequency and that the amount of interburst fluttering also increased, first in frequency and then in amplitude, until eventually on the sixteenth day of the 21-day period of adult development, the spiracular bursts disappeared and the spiracles fluttered continuously about a half-open position. It is important to note that in insects with very high metabolic rates the flutters had far greater amplitude than normal interburst flutters and resembled the spiracular movements encountered in the decline period after a spiracular burst. In any case, by using developing adults, it was possible to obtain animals with brief burst cycles. Another useful means of securing animals with brief cycles for convenient study was to injure pupae. Injury provokes a prompt increase in metabolic rate, which persists for days, and a tremendous increase in burst frequency (Schneiderman and Williams, 1955). In a typical experiment excision of the facial region from a Polyphemus pupa increased cycle frequency from one spiracular burst every two hours before injury, to 12 per hour four days after injury.

The explanation for these effects of metabolic rate on the spiracular burst cycle seems clear. One of the probable consequences of increased metabolic rate is to lower tracheal P_{O_2} and increase tracheal P_{CO_2}. It seems likely that these factors are directly responsible for the acceleration of the burst cycle. This is confirmed in the following experiments on spiracular occlusion.

3. Effects of spiracular occlusion

In pupal silkworms, the spiracles are the sole gateways to the tracheal system and their occlusion affords a simple method of lowering internal P_{O_2} and raising internal P_{CO_2}. The effects of spiracular occlusion on the cycle of valve activity of a Polyphemus pupa are seen in Figure 5. Sealing five pairs of abdominal spiracles with paraffin called forth an immediate increase in spiracular burst frequency from about one burst per hour to 6 or 8 per hour. After 12 or 13 of the 14 spiracles were sealed, the spiracular bursts disappeared completely. Unsealing the spiracles initiated the return to substantially normal behavior.

In pupae with five pairs of abdominal spiracles sealed and a spiracular burst frequency of 5 or 10 per hour, it was occasionally possible to restore normal burst frequencies of one to two per hour by exposing the pupa to pure oxygen. The increased oxygen also decreased interburst fluttering and led to wider openings at the time of the spiracular burst. The implications of these observations will be examined in the Discussion. For our present purposes suffice it to say that these spiracular occlusion experiments indicate that low oxygen combined with high carbon dioxide increases spiracular valve movements and speeds up the burst cycle.


A
"0"
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B
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10
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C
"TWELVE"
1 MIN.
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40
70
80
90
100
D
"THIRTEEN"
1 MIN.
10
20
30
40

FIGURE 5.

TABLE III

Effects of P_{O_2} on the various phases of the spiracular burst cycle of two diapausing Cecropia pupae (S-82 and S-93)

Expt. No.	Ambient P_{O_2}	Average cycle length (minutes)	Mean duration of various phases (minutes) Interburst*				Spiracular burst	
			Constriction	Percentage change in constriction period**	Flutter	Percentage change in flutter period**	Open	Decline
827	21	114	13 (11)	+462	91.5 (80)	−100	5.5 (4.8)	4.0 (3.5)
827a	100	75	73 (97)		0 (0)		2.1 (2.8)	0 (0)
938a	5	Continuous fluttering	—	—	—	—	—	—
939a	15	73	11.5 (16)	−36	58 (79)	+32	1.4 (1.9)	1.5 (2.1)
930 (Av.)	21	66	18.0 (28)	0	44 (65)	0	1.2 (1.9)	3.0 (4.7)
9310	35	50	28 (56)	+56	18 (36)	−59	2.0 (4.0)	2.0 (4.0)
9311	75	61	58 (95)	+220	0 (0)	−100	1.0 (1.6)	2.0 (3.3)

* Figures in parentheses record the *average percentage of the cycle length* occupied by the particular phase. Most of the results represent the average of two cycles except the results in air (930) which represent the average of five cycles of 50 to 74 minutes duration.
** Compared with air controls.

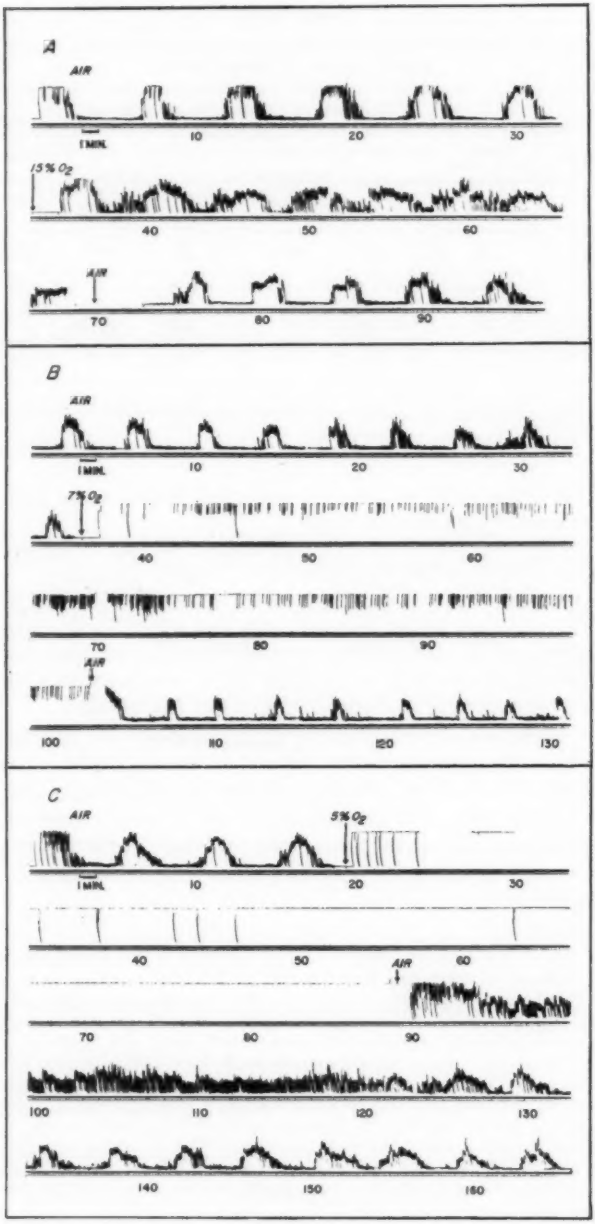
To separate the action of these gases on spiracular behavior, we tested the effects of specific gas mixtures on valve activity.

4. *Effects of oxygen tension on the spiracles*

The effects of decreased oxygen tension on spiracular behavior are evident in Figure 6. This pupa had a high metabolic rate and a brief burst cycle. As Figure 6A shows, within six minutes of lowering the ambient oxygen tension to *15%*, interburst fluttering increased so much that only a semblance of a burst cycle remained. The cycle returned to normal after about five minutes.

As Figure 6B shows, *7%* oxygen completely obliterated the burst cycle. Within one minute the valve opened widely without fluttering, remained open for five minutes with only an occasional closure, and then closed partially five to ten times each minute. Manifestly, this gradual return of spiracular fluttering looks like adaptation, but in our opinion it is more likely the result of a lowered P_{CO_2} within the insect as a result of prolonged spiracular opening (*cf.* Discussion). Within one minute of return to air the valve began to flutter closed and soon thereafter the burst cycle reappeared.

FIGURE 5. Effects of spiracular occlusion on the behavior of the second right abdominal spiracle of a Polyphemus pupa. Air at 25° C. (A) *No* spiracles sealed; 14 functional. A burst occurred just before the recording was begun. For 63 minutes thereafter no bursts occurred. (B) *Ten* spiracles sealed: first, third, fourth, fifth and sixth pairs of abdominal spiracles; four functional. Burst frequency increased from less than one per hour to one every 8 or 10 minutes. The pattern persisted essentially unchanged for 20 hours. (C) *Twelve* spiracles sealed: all of above plus both thoracic spiracles; two functional. Fluttering of valve in open position for periods of 8 to 20 minutes punctuated by brief one-half- to two-minute periods of normal fluttering about the closed position. (D) *Thirteen* spiracles sealed: all of above plus left second abdominal spiracle; one functional. Only the exposed spiracle was available for gas exchange. The result was intermittent closures from a wide-open position, first rapidly and then more slowly.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AIR
1 MIN.
10
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15% O2
40
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60
AIR
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B
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7% O2
40
50
60
70
80
90
AIR
100
110
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130
C
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5% O2
1 MIN.
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160

FIGURE 6.

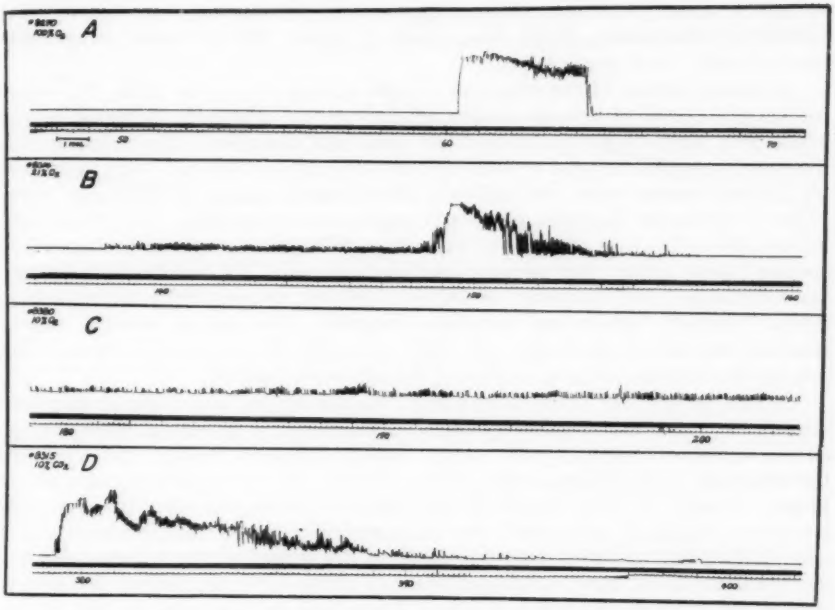

FIGURE 7. Records of spiracular movements of a Cecropia pupa in various ambient gas tensions. In all cases records were made when spiracular activity had reached a steady state. (A) 100% O_2; (B) air; (C) 10% O_2; (D) 10% CO_2. For details see text.

Figure 6C reveals that within 30 seconds of exposure to 5% oxygen, the valve opened widely with no initial fluttering. It gaped open for 70 minutes, closing at irregular intervals for about 40 minutes, and finally stayed wide open and motionless. Within one minute of return to air, fluttering began and continued for 40 minutes, whereupon the burst cycle reasserted itself. Recovery from 5% oxygen thus took nearly forty times longer than recovery from 7% oxygen, suggesting that this low oxygen tension was inadequate for respiration and caused the pupa to become anoxic and build up an oxygen debt. In *pure nitrogen,* a record similar to the 5% oxygen record was obtained (not illustrated). The spiracle closed occa-

FIGURE 6. Effects of P_{O_2} on the behavior of the second right abdominal spiracle of a diapausing Polyphemus pupa. Three records were obtained in studying each gas mixture: in air, in the specfic gas mixture, and again in air, to appraise the recovery of the spiracle. The time required to change the atmosphere in the chamber was less than 15 seconds. In this experiment and in succeeding ones, the reaction time of the spiracle depended very much on the phase of the burst cycle in which the gas was administered. As one would expect, the most rapid responses occurred when gases were admitted during a spiracular burst. In experiments illustrated the gases were added midway in the interburst period. (A) *15% O_2.* Record of valve movements in air, in *15% O_2 + 85% N_2,* and in air. (B) *7% O_2.* Record of valve movements in air, in *7% O_2 + 93% N_2,* and in air. (C) *5% O_2.* Record of valve movements in air, in *5% O_2 + 93% N_2,* and in air.

sionally for about 10 minutes, but then gaped widely, closing only once during thirty minutes of observation. From these results it appears that decreased oxygen tension can cause valve opening.

A clearer picture of the effects of oxygen tension is seen in Table III, which records the effects of several oxygen tensions on the spiracular behavior of two pupae with longer burst cycles than the pupa just described. These pupae took somewhat longer to respond to both high and low oxygen tensions, often more than 10 minutes except when the gas was administered during a spiracular burst. Figure 7 illustrates portions of several representative records. In *5% to 10% oxygen* the cycles were absent and the valves were continuously in motion. The openings were larger than normal interburst openings, but smaller than normal burst openings. The frequency of flutters, however, was not very different from typical interburst flutters and remained irregular. The period between volleys, however, was much decreased and rarely exceeded 20 seconds, whereas in air, two-minute intervals between volleys of flutters were common.

In *15% oxygen* the bursts reappeared. As the oxygen concentration increased above 15%, the width of valve openings in the interburst decreased, while the frequency remained essentially the same. Also, as oxygen tension increased, the fluttering period got progressively shorter and the constriction period progressively longer. Finally, in pure oxygen all the fluttering before and after the spiracular burst was completely eliminated: the valve remained closed until it abruptly shot open in the burst, and then almost as abruptly closed until the next burst. During the spiracular burst itself, at oxygen tensions above 15%, the higher the oxygen tension, the wider was the valve opening. From these results it appears that oxygen tension affects primarily interburst fluttering but also has some minor effects on the burst itself. It is also noteworthy that increasing or decreasing oxygen tension failed to have any predictable effect on burst frequencies: sometimes frequencies were lowered, other times increased.

TABLE IV

*Effects of P_{CO_2} on various phases of the spiracular burst cycle of a diapausing Cecropia pupa (No. S-83)**

Expt. No.	Ambient P_{CO_2}	Average cycle length (minutes)	Average duration of various phases (minutes)**					Percentage change in spiracular burst	Open	Decline
			Constriction	Percentage change in constriction period	Flutter	Percentage change in flutter period	Spiracular burst			
8311	Air	34	10 (29)	+30	21 (62)	−5	3.1 (9.2)	+62	1.4 (4.1)	1.7 (5.1)
	5%	38	13 (36)		20 (50)		5.0 (13.7)		1.8 (5.1)	3.2 (8.6)
8314	Air	50	16 (32)	+13	29.5 (59)	−81	4.5 (9.0)	+67	1.5 (3.0)	3.0 (6.0)
	8%	31	18 (59)		5.5 (18)		7.5 (24.0)		3.0 (9.7)	4.5 (14.3)
8315	Air	65	12 (19)	0	49 (75)	−91	4 (6.2)	+150	1.0 (1.6)	3.0 (4.7)
	10%	26	12 (45)		4.5 (17)		10 (38)		4.0 (15.0)	6.0 (22.5)
8316	Air	74	10 (14)	—	61 (81)	—	3 (4.1)	—	1.0 (1.4)	2.0 (2.7)
	15%	Continuous flutter	—		—		—		—	—

* Experiments were performed on successive days and the normal and experimental records for each day are paired. Most of the results represent the average of two cycles.

** Figures in parentheses record the *average percentage of the cycle length* occupied by the particular phase.

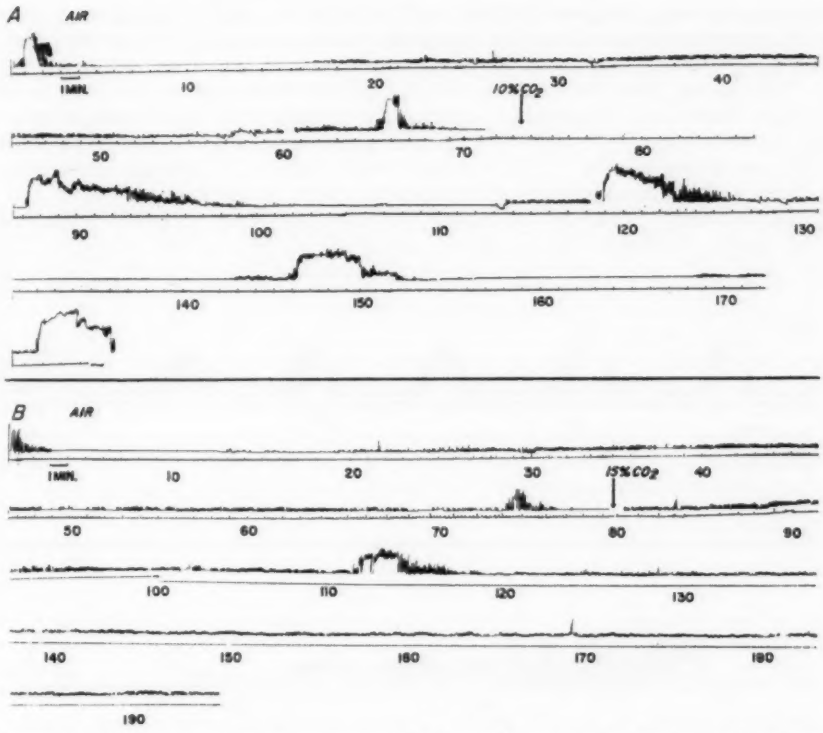

FIGURE 8. Records of spiracular movements of a Cecropia pupa with a moderately long burst cycle in air, followed by 10% or 15% CO_2. (A) 10% CO_2; (B) 15% CO_2.

In occasional experiments, particularly with pupae with high metabolic rates and frequent bursts, pure oxygen failed to suppress interburst fluttering. Instead the spiracular bursts disappeared and the spiracles fluttered continuously. Possible reasons for this curious behavior will be offered in the Discussion.

5. *Effects of carbon dioxide tension*

Increasing the P_{CO_2} increased burst frequency and lengthened both "open" and "decline" phases of the cycle (Table IV and Fig. 8). Besides the effect on burst frequency, the table and figure reveal that high tensions of carbon dioxide also lengthened both the open phase of the spiracular burst and the decline phase after the burst. Thus, in 10% carbon dioxide, the open phases were nearly three times as long as in air (compare Figure 7D with 7B). In addition, although increasing P_{CO_2} had little effect on the duration of the constriction period, it greatly shortened the flutter period until, in 10% carbon dioxide, fluttering was reduced to only a few minutes (Fig. 8). Between 10 and 15% carbon dioxide the burst cycle broke down completely, so that in 15% carbon dioxide the valves fluttered continuously (Fig. 8),

and after an initial spiracular burst, no further bursts occurred. The openings were a bit larger than normal interburst flutterings, but much smaller than normal openings in a burst.

The reaction times for a carbon dioxide response never appeared as long as the reaction times for a response to oxygen. Also, pupae with high metabolic rates, and hence frequent bursts, appeared far more sensitive to carbon dioxide than pupae with low metabolic rates. Thus, in a pupa which had a 4- to 5-minute burst cycle, as little as 2% carbon dioxide had a detectable effect on spiracular behavior (prolonged the burst), while 3% eliminated spiracular bursts and provoked continuous wide flutters. Furthermore, when the carbon dioxide tension was raised to 15% in a pupa with such a brief cycle, the valves opened widely, and nearly a half hour

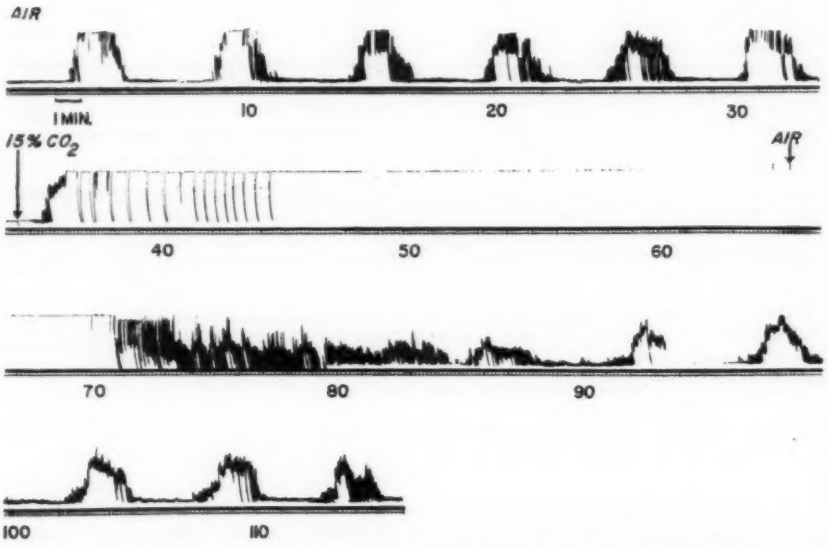

FIGURE 9. Records of spiracular movements of a Polyphemus pupa with frequent bursts in air, in 15% CO_2 + 21% O_2, and in air.

was required for recovery upon return to air (Fig. 9). Such sensitivity to carbon dioxide contrasts markedly with the more modest responses recorded in Figure 8, shown by a pupa with a longer burst cycle. A further observation important to our analysis is that in a pupa in which 3% carbon dioxide eliminated the spiracular bursts, bursts could be restored in 3% CO_2 by raising the oxygen tension to about 90%. This observation suggests that high oxygen tensions decrease the sensitivity of the spiracle to carbon dioxide, a fact to which we shall return.

Although these results convey a general picture of the effects of carbon dioxide and oxygen on the pupal spiracles, they suffer from a conspicuous defect: they provide only little information about the actual gas concentrations *within* the tracheal system that produce a particular effect. In other words, when the insect

is exposed to 15% oxygen or 10% carbon dioxide what are the actual intratracheal oxygen and carbon dioxide tensions? A partial solution to this problem was provided by experiments with intubated pupae which are described below.

B. Experiments With Intubated Pupae

1. *Effects of spiracular intubation*

Just as spiracular occlusion decreases the P_{O_2} and increases the P_{CO_2} within the pupal tracheal system, so spiracular intubation has the opposite effect. In the following experiment, the spiracular valves were exposed in four or five abdominal spiracles on one side of a series of diapausing Cecropia pupae. Into two, three or four of these spiracles short fire-polished glass tubes, about 1 mm. in diameter, were inserted beyond the spiracular valves, placing the tracheal system in free communion with the air. This maneuver caused the P_{CO_2} within the insect to fall and the P_{O_2} to rise, as the gaseous composition of the tracheal system approached that of the outside.

One or two days after the operation, and periodically thereafter for about a week, the unintubated spiracles were examined (usually the second and third abdominal). In every case the spiracular valves appeared to stay tightly constricted for many days. They could be opened, however, by exposing the insect to low oxygen or high carbon dioxide. Thus, although the untouched spiracles of the intubated pupa could still function, their cyclical activity had disappeared, presumably because the normal triggering stimuli for spiracular activity were absent.

When intubations were performed on animals with very high metabolic rates (*e.g.* developing adults), despite four intubated spiracles some spiracular fluttering occurred. Apparently, intubating even four spiracles in animals with high metabolic rates does not permit equilibration of internal and external gases.

These experiments, coupled with the other experiments considered thus far, are consistent with the view that the triggering stimulus for spiracular opening is either low internal tension of oxygen, high internal tension of carbon dioxide, or a combination of the two. This matter is considered in further detail below.

2. *Effects of oxygen tension on the spiracles of intubated animals*

Intubation eliminates the spiracular burst cycle and the spiracles exhibit a persistent pattern of behavior in various gas mixtures. Thus, the intubated pupae provide a simple means of determining the approximate concentrations of oxygen and carbon dioxide that produce a particular kind of spiracular behavior, *e.g.* valve fluttering, bursts etc., and also a means of examining the interaction between carbon dioxide and oxygen in controlling spiracular movement. To conduct these experiments, pupae with two or three intubated spiracles were exposed to various gas mixtures in a flow system, and the movements of two normal spiracles observed until they exhibited a constant behavior. It took anywhere from 5 to 35 minutes for the constant response to assert itself, except in very low oxygen tensions or very high carbon dioxide tensions where the response appeared more rapidly. The decision to study the final response of the spiracles rather than their initial response was taken for several reasons. The principal one was that, in these large insects, it very likely takes many minutes for gases to reach a steady-state. A second

TABLE V

*Determination of the "O_2-open" threshold of two spiracles of an intubated Cynthia pupa**

Per cent oxygen	Response of spiracle 2R	Response of spiracle 3R
21	Closed	Closed
15.5	Active fluttering	Closed
8.5	Partly open with rapid closures	Slight fluttering (pulsation)
5.0	Wide open with occasional flutters	Active fluttering
4.5	Wide open with occasional flutters	Active fluttering
4.0	Wide open with occasional flutters	Wide open with occasional flutters
3.5	Wide open with occasional flutters	Wide open with occasional flutters
3.0	*Wide open with no closures*	Wide open with occasional flutters
2.5	*Wide open with no closures*	Wide open with occasional flutters
2.0	*Wide open with no closures*	*Wide open with no closures*
1.5	*Wide open with no closures*	*Wide open with no closures*

* Almost identical results were obtained when the gas mixtures were flushed through the chamber at random instead of in order, provided adequate recovery periods were allowed. Hence, under the conditions of our experiments, previous exposure to a test gas mixture had no effect on subsequent tests.

reason was that, in the normal intact insect, gas changes are ordinarily gradual and not cataclysmic, and hence it seemed a sensible procedure to allow time for adjustment to each change of gas.

Table V illustrates the data recorded to determine the effects of oxygen on two spiracles of a typical diapausing pupa. Progressive lowering of the ambient oxygen tension caused in sequence: pulsation of the valve (no visible opening), fluttering (small openings), partial openings and closings, full opening and occasional closure, and finally full opening with no movement, *i.e.*, complete relaxation of the spiracular closer muscle.

TABLE VI

"O_2-flutter" thresholds and "O_2-open" thresholds of abdominal spiracles of five intubated Cecropia pupae

Experiment No.	Spiracle observed	"O_2-flutter" threshold* (per cent)	"O_2-open" threshold** (per cent)
21 b	1 L	12	3
	3 L	8.5	3
22 b	1 L	12	1.5
	2 L	12	1.5
23 b	2 L	8.5	1.5
	3 L	5.5	1.5
24 b	1 L	12	1.5
	2 L	8.5	1.5
25 b	2 L	5.5	1.5
	3 L	5.5	1.5

* P_{O_2} which caused first pulsations.
** P_{O_2} which caused full spiracular opening for one minute.

Two oxygen tensions are of special interest: the tension that initiates spiracular movement, *i.e.*, pulsations or fluttering, and the tension that causes a spiracle to open fully, as in a burst. A typical set of values for these two oxygen tensions for a series of diapausing pupae is recorded in Table VI. From the table it can be seen that the five pupae studied varied considerably in their sensitivity to oxygen. Spiracular movements began at oxygen tensions ranging from 12 to 5.5%. The tensions required for full opening were more uniform. Unfortunately, the oxygen tension that induced pulsations proved to be exceedingly difficult to determine since it was not reproducible in successive experiments. The oxygen tension that produced full opening of the spiracle was far simpler to ascertain. Therefore, to facilitate quantitative measurements we concentrated on one spiracular response: full opening of the valve for one minute. This response occurs normally during a burst cycle, was easily judged, and was altered by changes in oxygen or carbon dioxide tensions of $\pm$ 0.5%. Moreover, it was easily reproducible.

The tension of oxygen necessary to open the spiracles widely was determined for 33 spiracles of more than a dozen Cynthia pupae that had several spiracles intubated. This tension of oxygen, hereafter termed the "O_2-open threshold," averaged 2.58 $\pm$ 1.12(s.d.)% with a range of 1% to 5.5%. Two-thirds of the spiracles responded to between 2% and 3.5% oxygen. Within any pupa the O_2-open threshold for

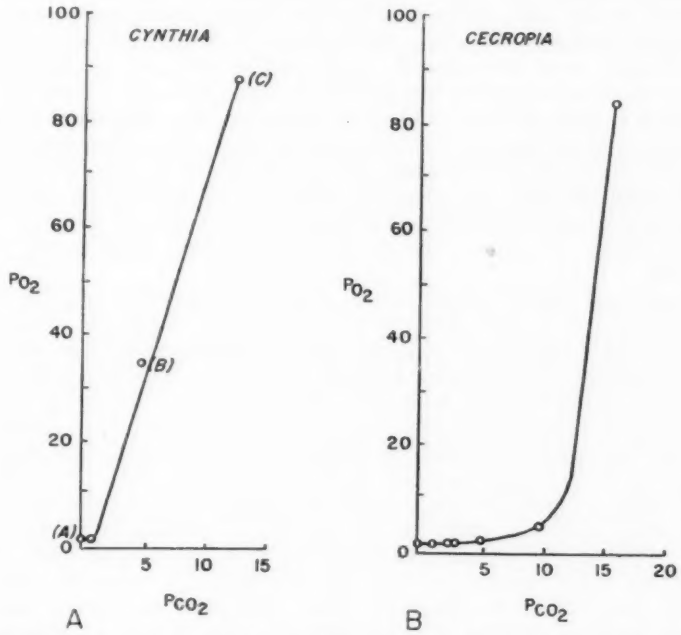

FIGURE 10. (A) The effect of P_{O_2} on the P_{CO_2} necessary to open a spiracle of an intubated Cynthia pupa widely for one minute. Partial pressures are expressed as % atm. For details see text. (B) Same curve as above for an intubated Cecropia pupa.

different spiracles did not vary by more than 1.5%. For a given spiracle, at all oxygen tensions below threshold the spiracle was wide open and the spiracular muscle remained relaxed; at tensions 0.5% or more above the O_2-open threshold, intermittent to prolonged contractions of the muscle occurred, which closed the valve (*cf.* Table V). The O_2-open threshold was also determined for a few developing adults of Polyphemus and found to average 3.1% oxygen with a range of 2.0–4.5%.

3. *The interaction between oxygen and carbon dioxide in the spiracles of intubated animals*

The carbon dioxide tension necessary to open the spiracle widely for one minute varied with the oxygen tension. This is seen in Figure 10A which records the behavior of a Cynthia pupa. The first point (A) on the curve gives the highest partial pressure of oxygen in a nitrogen-oxygen mixture that kept the spiracle open, *i.e.*, the O_2-open threshold which we considered in the previous section. The third point (B) on the curve was determined by adding a mixture of 4% carbon dioxide to air, a mixture which kept the spiracle wide open, and progressively increasing the oxygen tension until the spiracle began to close. In this experiment, in the presence of 4% carbon dioxide, oxygen tensions above 35% were necessary to close the spiracle. In other words at any point along the curve for a given partial pressure of carbon dioxide there was a critical tension of oxygen, below which the valve would remain fully open and motionless; oxygen tensions above this critical tension caused closure of the valve. The end-point of this curve is (C). At carbon dioxide tensions above this value the valve remains open and motionless and no partial pressure of oxygen at atmospheric pressure will cause fluttering. This point, which is designated the "CO_2-open threshold," was determined by starting in pure oxygen where the valve was closed and increasing the carbon dioxide tension until a tension was reached at which the spiracle remained open for one minute. This CO_2-open threshold was simple to determine and reproducible within about 1%. Several curves of this sort were recorded for a number of pupae and all looked essentially the same. One typical of a Cecropia pupa is presented in Figure 10B.

Although such curves are difficult to obtain, by measuring only the O_2-open threshold and the CO_2-open threshold of a spiracle it was possible to assess in a general way the sensitivity of the spiracle and to compare spiracles of pupae under various conditions. This was done for a series of Cynthia spiracles and the O_2-open thresholds have already been discussed. The CO_2-open thresholds of this same series of spiracles averaged 17.63 ± 5.42(s.d.)% and covered a far greater range (13.5% to 31%) than the O_2-open thresholds. Notwithstanding, two-thirds of the spiracles had a CO_2-open threshold between 14% and 16%. The CO_2-open threshold appeared quite uniform for different spiracles of any individual. A few determinations were also made on the spiracles of developing adults of Polyphemus. Whereas the O_2-open thresholds for these insects with high metabolic rates did not differ significantly from the thresholds for diapausing pupae, the CO_2-open thresholds of the developing adults were lower than for the diapausing pupae and averaged only 10.5% with a range of 9.5–12.0%. Furthermore, the CO_2-open thresholds decreased on successive days of adult development as the metabolic rate increased.

These results demonstrate the influence of oxygen on the response of the spiracle to carbon dioxide, and indicate that the sensitivity to carbon dioxide decreases as oxygen tension increases. Similar results were obtained when the carbon dioxide tension that caused fluttering was determined: the sensitivity to carbon dioxide decreased as the oxygen tension increased.

Discussion

1. *Scope of the analysis*

The spiracular behavior of giant silkworms just described represents one of the most extreme kinds of spiracular behavior recorded for any insect, with a complex cycle of activity which commonly lasts for hours and occasionally for days. The present experiments confirm and amplify earlier opinions that the cyclical behavior of the spiracles is somehow responsible for discontinuous respiration. They may have, in addition, some intrinsic interest for insect respiration as a whole. For, in a sense, these giant pupae breathe in "slow motion," and events which may occupy only a second or two in the respiratory cycles of other insects may take many minutes or even hours. This enables the experimenter to analyze complex events in gas exchange and spiracular behavior which are not readily separable in insects with brief cycles. The present experiments have exploited this property in an effort to define the kinds of behavior that spiracles can show, and to clarify the manner in which oxygen and carbon dioxide interact to provoke various modes of spiracular activity.

2. *The behavior of the spiracular muscle*

Interpretation of the present experiments is simplified by the fact that the movements of each pupal spiracle are controlled by a single muscle. Thus, the records of spiracular activity are records of the behavior of this muscle. As we have seen, the muscle displays three sorts of behavior: sustained contraction which causes valve closure; brief partial relaxation which causes valve fluttering; and almost complete relaxation which causes the valve to gape open.

In the normal pupa these different kinds of behavior occur in two characteristic patterns: (1) the first is the "flutter," in which the muscle relaxes slightly and then quickly re-contracts fully. This occurs many times each minute in irregular volleys and may continue for hours. It is important to note that the relaxations are very brief and that during most of the flutter period the valves are constricted. Indeed, as far as one can tell, they are as tightly constricted between flutters as they are during the constriction period after a burst. (2) The second pattern, the "burst" cycle, is more dramatic and far more regular. It is compounded of numerous flutters and periods of prolonged valve opening and prolonged constriction.

The initiation of each of these patterns of spiracular behavior does not appear to depend upon an endogenous nervous rhythm as is true, for example, in the respiratory movements of *Schistocerca* and *Dixippus* (*cf.* review by Wigglesworth, 1953a, and also Hoyle, 1959) or of vertebrates, but is the result of cyclical changes in the composition of the tracheal gases. The mechanism of this extrinsic control is described below.

3. *An explanation for the burst cycle*

All events in the normal burst cycle, as well as the experimental observations, can be explained in terms of the following three properties of the spiracular muscle and its associated nerves: (a) the muscle is ordinarily continuously stimulated to contract; (b) when tracheal P_{O_2} falls below a critical value, the flutter threshold, the muscle repeatedly relaxes slightly and re-contracts; (c) when tracheal P_{CO_2} rises above a critical value, the burst threshold, the muscle relaxes fully. These assertions permit us to frame the following hypothesis which appears to be consistent with all of our observations and accounts for the burst cycle.

Immediately after a burst the valves are constricted, tracheal P_{O_2} is far above the flutter threshold, and tracheal P_{CO_2} is far below the burst threshold. This is the constriction period. As the insect respires, oxygen inflow fails to match oxygen utilization and the P_{O_2} within the tracheal system falls. When the tracheal P_{O_2} falls to about 3%, the flutter threshold, spiracular fluttering begins, permitting oxygen to enter. This increases the tracheal P_{O_2} and the valve promptly closes. This pattern of rapid fluttering, punctuated by short periods of constriction, represents the flutter period. In high oxygen tensions tracheal P_{O_2} never reaches triggering concentration and hence no fluttering occurs.

What about the spiracular burst itself? This involves carbon dioxide. During the interburst period some carbon dioxide escapes, but at a rate considerably less than that of its production. As a consequence, internal P_{CO_2} ultimately reaches the spiracular burst threshold, and the spiracular muscle relaxes. The valve opens widely in a burst; carbon dioxide and nitrogen flow out and oxygen flows in, as the composition of the tracheal gas approaches that of the atmosphere. When the internal P_{CO_2} decreases below a certain value, the valve begins to open and close periodically, with diminishing width and duration of opening, until it finally remains constricted and the cyle is completed.

Detailed support for this hypothesis and for the assertions regarding the effect of P_{O_2} and P_{CO_2} on the spiracular muscle are examined below.

4. *Oxygen effects: the constriction and flutter periods*

A number of experiments prove that fluttering is caused by low tracheal P_{O_2} and is little affected by tracheal P_{CO_2}. Firstly, in a normal pupa, lowering ambient P_{O_2} to about 10% evokes continuous valve fluttering (presumably because tracheal P_{O_2} is lowered). Secondly, raising ambient P_{O_2} suppresses entirely the valve fluttering of a normal pupa: apparently under this condition tracheal P_{O_2} never falls to the flutter threshold. Thirdly, when several spiracles are intubated, thereby raising the internal P_{O_2}, fluttering ceases. Finally, in such an intubated pupa, when ambient P_{O_2} is lowered to about 2% fluttering continues indefinitely.

Under this analysis, in a normal pupa the *duration of the constriction period* after a burst is determined primarily by the rate at which P_{O_2} falls within the tracheal system. This in turn depends upon three factors: (1) the tracheal volume of the pupa, (2) the rate of oxygen utilization by the pupa, and (3) the rate at which oxygen leaks in through the constricted spiracles. In general, large pupae with low metabolic rates have long constriction periods. Also, as one would predict, the constriction period is prolonged by increasing ambient P_{O_2} (*cf.* Table III),

for at the end of a burst, not only is there a higher O_2 content in the tracheal system, but also more O_2 leaks inward.

In occasional experiments, pure oxygen failed to suppress valve fluttering but instead abolished the burst cycle. Similar results were found by Buck *et al.* (1955) in manometric experiments with *Agapema* pupae. Neuromuscular mechanisms of insects are particularly susceptible to oxygen poisoning (Goldsmith and Schneiderman, 1960) and this may account for these results.

What determines the frequency of fluttering and the duration of the closed period between each volley of flutters? Both of these appear to be governed, as is the duration of the constriction period, by the rate at which P_{O_2} falls within the tracheal system, and therefore depend in part upon metabolic rate and upon ambient P_{O_2}. As one would expect, the higher the metabolic rate, the more frequent the flutters and the more frequent the volleys of flutters (*cf.* Figures 4 and 6). Moreover, since the frequency of the flutters and the frequency of volleys depend primarily upon tracheal P_{O_2}, it is not surprising that they do not change systematically during the course of the flutter period, for *once fluttering begins, tracheal P_{O_2} presumably varies very little since oxygen continuously enters.* This prediction has been confirmed by analyses of tracheal gas composition (Levy and Schneiderman, 1957, 1958), which will be discussed in detail in a subsequent report. For our present purposes this constant P_{O_2} is of singular importance, for it tells us that the termination of the flutter period by a spiracular burst depends not upon a change in tracheal P_{O_2}, but upon the accumulation of carbon dioxide. This is considered below.

5. *Carbon dioxide effects: "triggering" and termination of the spiracular burst*

The most conspicuous effect of an increase in ambient P_{CO_2} was the shortening of the burst cycle, which confirms previous manometric findings (Schneiderman and Williams, 1955). This reduction in cycle length was largely at the expense of the flutter period, which was greatly shortened, whereas the duration of the constriction period was unchanged. In short, increasing ambient P_{CO_2} led to the premature triggering of the burst early in the flutter period. Moreover, once triggered, the burst was prolonged. These observations can be understood in the following terms:

(1) *The absence of an effect of carbon dioxide on the duration of the constriction period* supports the opinion offered above, that in the intact pupa the onset of fluttering is triggered primarily by low tracheal P_{O_2}. Further support comes from the fact that the P_{O_2} which triggered off the fluttering was not significantly affected by modest increases in tracheal P_{CO_2}. Indeed, in pupae with long cycles, until ambient P_{CO_2} rose above 10%, it did not appear to affect the amplitude or frequency of flutters.

(2) *The premature triggering of spiracular bursts by ambient P_{CO_2}.* A variety of experiments revealed that in a normal burst cycle, the spiracular bursts themselves are triggered by tracheal P_{CO_2}, and not by tracheal P_{O_2}. Thus exposing both normal and intubated pupae to physiological and non-anaesthetic concentrations of carbon dioxide causes the spiracular valves to open widely. In a normal cycle, tracheal P_{CO_2} gradually increases after a burst as a result of metabolism and, when it reaches a certain level, the spiracular muscle fully relaxes and a burst occurs. At *all* concentrations of carbon dioxide, tracheal P_{CO_2} more quickly reaches the

triggering concentration, because the partial pressure gradient driving carbon dioxide out of the insect is decreased, with a consequent decrease in the rate of carbon dioxide leakage. In addition, *high* ambient concentrations of carbon dioxide (*i.e.*, above 5%) prevent tracheal carbon dioxide from falling to its normal low level after a burst. This constant high level of tracheal carbon dioxide also shortens the time required to reach a trigger value. Thus, for these two reasons, the higher the ambient carbon dioxide concentration, the shorter the time between bursts. The second reason is by far the more important in pupae with low metabolic rates. In such insects ambient carbon dioxide tensions below 5% had no detectable effect on burst frequency, whereas increasing carbon dioxide from 5% to 10% often increased the burst frequency 10 to 20 times (Schneiderman and Williams, 1955). However, in pupae with high metabolic rates and high burst frequencies, the reduction in carbon dioxide escape caused by the presence of even 1% ambient carbon dioxide was often sufficient to abolish the bursts entirely.

A different kind of evidence for carbon dioxide triggering spiracular bursts comes from experiments on *Agapema* pupae, in which there was demonstrated an inverse relation between ambient P_{CO_2} and the *delay* in the first subsequent spiracular opening (Buck and Keister, 1958). Pupae in 9% carbon dioxide opened their spiracles 2¾ hours after exposure, whereas in 24% carbon dioxide they took only one hour. As the authors point out, this is what would be expected "if the carbon dioxide were leaking into the tracheal system and accelerating the rise to triggering concentration" (p. 335).

(3) *Further evidence for the triggering of spiracular bursts by tracheal* P_{CO_2}. The triggering of bursts by tracheal P_{CO_2} is well seen in 100% oxygen, where internal P_{O_2} might be expected to remain relatively high throughout the cycle. Here it seems clear that increasing tracheal P_{CO_2} was the trigger that caused the spiracular muscle to relax. The converse result is seen in intubated pupae, where one prevents carbon dioxide from reaching a concentration high enough to trigger a spiracular burst and the valves remain permanently closed.

(4) *Termination of the spiracular burst.* Once triggered to open in a burst, the spiracle remains open and carbon dioxide diffuses out until tracheal P_{CO_2} falls to some critical level, whereupon the spiracle closes again. Increased ambient P_{CO_2} prolonged bursts, presumably because it delayed the diffusive outflow of carbon dioxide. It is worth recalling that it takes the blood and tissues some time to unload carbon dioxide. In most cases, long before this is done the tracheal P_{O_2} has risen to its maximum value as oxygen diffuses in at the burst. Hence, the closing of the spiracle at the end of a burst has much less to do with tracheal P_{O_2} than with tracheal P_{CO_2}.

It is puzzling that immediately the carbon dioxide tension falls somewhat, the muscle does not contract again, but instead remains relaxed for many minutes, even when carbon dioxide tension has fallen well below the triggering threshold. In some way, high P_{CO_2} provokes a response in the muscle which is sustained for a considerable period (*cf.* discussion of this point by Buck, 1957). This sharply contrasts with the partial relaxations induced by low P_{O_2}, which are very transitory, and suggests that flutters and spiracular bursts are fundamentally different responses.

6. *Effects of metabolic rate*

The effects of metabolic rate on spiracular activity appear to be similar in most insects: high metabolism leads to increased spiracular movement (*cf.* review by Wigglesworth, 1953a). For example, Nunome (1944) has reported that the spiracles of *Bombyx mori* open more frequently and more widely when larvae were active than when they were inactive. In the present experiments, spiracular burst frequency increased with metabolic rate, confirming manometric studies. This increase in frequency is largely the result of more rapid production of carbon dioxide which shortens the time required for tracheal P_{CO_2} to reach triggering concentration. The more rapid decrease in tracheal P_{O_2} which attends high metabolism probably has only a limited impact, inasmuch as once fluttering begins, tracheal P_{O_2} remains constant.

When metabolic rate gets very high and bursts occur every 5 or 6 minutes (see for example Figure 6), the constriction period is obliterated, and between bursts the valves are in continuous motion, *i.e.*, fluttering. In effect, the decline period after the burst fuses with the flutter period before the next burst. This suggests that oxygen utilization is so rapid that even during the decline period tracheal P_{O_2} falls rapidly to the flutter threshold. When metabolic rate gets very high and the cycles last for less than four minutes, as is the case with late developing adults, the interburst flutterings increase markedly in amplitude until the burst cycles disappear entirely and are replaced by continuous wide flutters. As mentioned previously, these flutters are far larger than oxygen-induced flutters. It appears likely that when metabolism gets high enough to cause continuous fluttering, carbon dioxide may be viewed as the principal stimulus. Further support for this opinion comes from the observation that in intubated pupae the O_2-open threshold did not vary with metabolic rate, whereas the CO_2-open threshold decreased as metabolic rate increased. This suggests that in pupae with high metabolic rates, there is a considerable accumulation of carbon dioxide even when several spiracles are open. This high tracheal P_{CO_2} probably accounts for the fact that intubated pupae with very high metabolic rates showed continuous valve fluttering.

7. *Interaction between carbon dioxide and oxygen*

In the above analysis we have considered oxygen and carbon dioxide as acting essentially independently. This is not the case, but, as we shall see, this fact does not impair the analysis. Evidence that oxygen tension affects the carbon dioxide threshold was provided by the observation that high oxygen tension markedly reduced the burst frequency from 5 to 10 bursts per hour to one to two per hour in a pupa in which 5 pairs of spiracles were sealed. This finding suggests that the high oxygen tension raised the carbon dioxide threshold so that more carbon dioxide had to accumulate to cause a spiracular burst. A further piece of circumstantial evidence was the discovery that very high oxygen tensions often restored bursts in pupae in which carbon dioxide had eliminated the bursts. However, interpretation of these experiments was hampered by the fact that it was difficult to be certain of the tracheal gas composition, which varied continuously. Part of this difficulty was by-passed in experiments with intubated pupae. To be

sure, even in intubated pupae the tracheal gas composition is not known with certainty, but at least it can be kept constant and the oxygen and carbon dioxide tensions can be varied independently.

The intubation experiments reveal that the concentration of carbon dioxide necessary to open the spiracles wide varies with the oxygen tension. In the presence of 2.3% oxygen, 5% carbon dioxide opened the spiracles of a typical Cecropia pupa, whereas in 4.7% oxygen, 10% carbon dioxide was required. This result demonstrates clearly the interaction between P_{O_2} and P_{CO_2} in controlling the wide opening of spiracles, and it finds several parallels in the literature. Thus, in the cockroach (Hazelhoff, 1926a, 1926b), the flea (Wigglesworth, 1935), and in various muscid flies (Case, 1956a), the opening of spiracles in response to carbon dioxide is favored by low oxygen and depressed by high oxygen, just as in Cynthia and Cecropia pupae.

In the present experiments it is of some interest that, although P_{O_2} influenced the P_{CO_2} necessary to cause a spiracular burst, there did not seem to be a similar interaction in the triggering of flutters. Thus, as discussed in Section 5 above, increasing ambient P_{CO_2} up to 10% had no significant effects on the oxygen flutter threshold. Hence, it seems unlikely that during a normal burst cycle internal P_{CO_2} affects the oxygen flutter threshold, inasmuch as tracheal P_{CO_2} in these pupae rarely rises above 6.5% (Buck and Keister, 1958; Levy and Schneiderman, 1958).

It is not our present purpose to explore the mechanism of the interaction between oxygen and carbon dioxide in triggering bursts. However, recognition of this phenomenon enables us to interpret several events in the burst cycle to which we have not yet attended, notably the onset of the decline period. Why does the relaxed spiracular muscle start contracting again about one-third of the way through a burst? This might be the result of falling tracheal P_{CO_2}, rising tracheal P_{O_2}, or both. Evidence that it is largely rising P_{O_2} comes from records of pupae in pure oxygen. Here there is no decline period and the spiracle simply shuts. This suggests that under normal conditions the decline period is related to tracheal P_{O_2}. A possible mechanism might be the following.

During a spiracular burst, tracheal P_{O_2} equilibrates with the atmosphere far more rapidly than does tracheal P_{CO_2} because of the long time required to unload dissolved carbon dioxide. In a relatively short time, tracheal P_{O_2} rises to a concentration that increases the carbon dioxide burst threshold. The valve shuts briefly, to reopen promptly as carbon dioxide continues to accumulate in the tracheal system. Under this analysis, the onset of the decline period marks the point in a spiracular burst where tracheal P_{O_2} has risen high enough to depress appreciably the sensitivity of the spiracle to carbon dioxide. As mentioned in Section 5 above, the duration of the decline period is determined by the time necessary to unload carbon dioxide.

A second instance of O_2-CO_2 interaction appears to occur in the period just before a spiracular burst. Ordinarily, 20 to 120 seconds before a burst, flutters are larger than normal and gradually build up to the burst. This brief build-up period resembles the decline period and is also absent in pure oxygen, suggesting that, like the decline period, it reflects O_2-CO_2 interaction. The mechanism of this interaction will be considered subsequently, but for the present we can conclude that at the low P_{O_2} which obtains in a normal pupa just prior to a burst, the

carbon dioxide threshold of different excitatory elements is not as uniform as in high P_{O_2}.

Interaction between oxygen and carbon dioxide also explains why variations in ambient P_{O_2} have no predictable effect on burst frequency. High ambient P_{O_2} raises the carbon dioxide threshold of the spiracles, and this tends to decrease the burst frequency. But, simultaneously, high oxygen decreases interburst fluttering and this, in turn, decreases the rate of interburst carbon dioxide release, and increases the rate of carbon dioxide accumulation. This tends to increase the burst frequency. The ultimate outcome, as we know from manometric experiments (Schneiderman and Williams, 1955) and from the present study, is sometimes increased frequency, sometimes decreased frequency, and in other cases no change.

Table VII summarizes the conclusions reached in this discussion relating to the effects of tracheal P_{O_2} and P_{CO_2} on the behavior of the spiracular valves during the burst cycle.

TABLE VII

Summary of the effects of tracheal P_{O_2} and P_{CO_2} on the behavior of the spiracular valves during a burst cycle

Tracheal composition	Constriction period		Flutter period		Burst	
	Initiate	Terminate	Initiate	Terminate	Initiate	Terminate
Low P_{CO_2}	+	0	0	0	0	+
High P_{CO_2}	0	(+)	(+)	+	+	0
Low P_{O_2}	0	+	+	(+)	(+)	0
High P_{O_2}	±	0	0	0	0	±

\+ Principal agency responsible.
± Secondary factor.
(+) These conditions do not normally prevail in the phase of the burst cycle indicated.

8. *The existence of an independent oxygen-sensitive mechanism*

It is not a principal purpose of the present report to discuss the mechanisms whereby oxygen and carbon dioxide exert their effects, nor to consider the sites of action of these gases. Earlier experiments on these pupae ruled out the existence of any indispensable respiratory center (Schneiderman and Williams, 1953b; Schneiderman and Beckel, 1954; Schneiderman, 1956). They also showed that although nervous control was involved in the normal stimulation and coordination of the spiracular muscles (Schneiderman, 1956), the immediate response to carbon dioxide resided in the spiracular muscle itself (Beckel and Schneiderman, 1956, 1957). However, the present studies appear to clarify one point which has hitherto not been resolved for any insect, namely that, at least in silkworm pupae, *oxygen and carbon dioxide affect the spiracle via very different mechanisms.* It has been suggested by various workers that oxygen-lack produces acidity, and this might be its mode of action in stimulating spiracles. Wigglesworth (1935, 1953b) argues that in the flea both O_2-lack and CO_2-excess act in virtue of the

acidity they produce. Case (1957a) and Punt *et al.* (1957), on the other hand, raise the possibility that the acidity produced by oxygen-lack might release bound carbon dioxide from the blood and that CO_2 is the effective agent to which spiracles respond in both O_2-lack and CO_2-excess. In the case of the silkworm, the experimental results forbid either interpretation, as the following considerations show.

During the course of a burst cycle, when P_{O_2} falls to a critical threshold, fluttering begins and may continue uniformly for many hours. This sustained pattern of behavior in response to low P_{O_2} indicates that the primary response to oxygen lack is not acidity itself, nor acidity releasing bound carbon dioxide. For, during the entire course of the flutter period, acidity increases steadily as carbon dioxide accumulates. Notwithstanding, the flutter response did not change appreciably, nor did it change after ambient P_{CO_2} was raised to 10% (*cf.* Figure 8 and Section 5 above), a maneuver which certainly increased acidity. These observations suggest that, in the silkworm, increasing acidity is probably not the means whereby oxygen lack makes itself felt. They also seem to prove that in the silkworm pupa there is a specific mechanism sensitive to oxygen lack that may act independently of any carbon dioxide-sensitive mechanism.[4] Evidence will be presented in a subsequent paper that P_{O_2} may act on the central nervous system in contrast to P_{CO_2} which acts peripherally.

9. *Comparison with the flea*

In concluding, it is fruitful to compare the spiracular behavior of these silkworm pupae with the behavior of the spiracles of the flea described by Wigglesworth (1935) in his fundamental study. The flea spiracle is operated by a single closer muscle and shows a simple pattern of opening and closing. In air at 20 to 22° C. cycles are 12 to 16 seconds long, 6 to 8 seconds during which the spiracle stays closed followed by about an equal time during which the spiracle is open. P_{O_2} and P_{CO_2} affected this cycle in a predictable way. As P_{O_2} diminished from 100% there was an increase in cycle frequency; the closed period was considerably shortened, whereas the open period was less affected. As P_{CO_2} increased, the open period was lengthened considerably, whereas the closed period was slightly shortened. It was concluded that the spiracles were caused to open chiefly by falling P_{O_2}, rising P_{CO_2} being less important.

In the pupal burst cycle, the constriction period corresponds to the closed period in the flea, and the spiracular burst corresponds to the open period in the flea. Because the flea has no flutter period we cannot compare the effects of various gas mixtures on the length of an entire cycle (*i.e.*, on the frequency of the cycles), but we can compare the periods of valve closure and of valve opening in both insects.

(a) *Oxygen effects*

In both the flea and the pupa the duration of the constricted period is proportional to P_{O_2}, and in both, the spiracle is caused to open by low P_{O_2}. However,

[4] Very recently Hoyle (1960) has provided convincing evidence that in the spiracular muscle of the locust, CO_2 acts directly on the neuromuscular junction and blocks transmission of nervous excitation. This important discovery supports the view outlined above that CO_2 acts peripherally.

unlike the flea spiracle, which opens widely and stays open for a number of seconds after a constriction period, the pupal spiracular muscle relaxes only slightly when a critical P_{O_2} is reached and then re-contracts immediately. This behavior is repeated over and over during the flutter period, for which there is no counterpart in the flea. In the pupa, for reasons already discussed in Section 7, P_{O_2} has no predictable effect on cycle frequency.

(b) *Carbon dioxide effects*

In both the flea and the pupa the duration of the spiracular burst or open period is proportional to P_{CO_2}. And in both, P_{CO_2} has only small effects on the duration of the constriction period. It is noteworthy that in the flea, carbon dioxide fails to affect the frequency of openings and in the pupa it fails to affect the frequency of flutters or volleys of flutters in the flutter period. These observations support the view that the open period of the flea and the flutter period of the pupa are largely triggered by low tracheal P_{O_2}, and that tracheal P_{CO_2} is ordinarily of much less importance in this connection. It is nonetheless of some significance that, in the flea, raising the P_{CO_2} decreased the closed period, albeit only slightly. In commenting upon this, Wigglesworth (1935) remarks (p. 405) that the closed period of the flea is terminated "chiefly by oxygen want (carbon dioxide contributing to a small extent)." The pupal spiracular burst cycle enables us to separate clearly the oxygen-want effect from the carbon dioxide effect, for in the pupa the oxygen-want effect—fluttering—not only appears first, but is qualitatively different from the carbon dioxide effect—the initiation of a spiracular burst. In only one place in the flea are these effects separable, namely, in pure oxygen. Here, in the flea as in the pupa, the spiracular bursts seem to have been triggered mainly by P_{CO_2} and not by oxygen lack. It is also noteworthy that in both the flea and the pupa, the effects of carbon dioxide varied directly with the intensity of metabolism.

A further point of similarity between the flea and the pupa is that in both, the denervated spiracular muscle remains contracted (Wigglesworth, 1935; Schneiderman, 1956). In the case of the pupal silkworm the muscle remains contracted for many weeks, and this appears to be true also of the bed-bug (Wigglesworth, 1941) and the roach (Case, 1956b, 1957b). These are all insects without nervous-controlled ventilation movements under ordinary conditions. By contrast, the spiracular muscles of insects like *Schistocerca*, which have ventilation movements, relax when denervated (Fraenkel, 1932; Hoyle, 1959). Indeed, it may be that in many insects where the rhythmical activity of spiracles is under nervous control, denervation leads to relaxation, whereas in others, like the flea and silkworm pupae, where the spiracles are under the extrinsic control of respiratory gases, the denervated spiracular muscle continues to contract.

In sum, it appears that the behavior of the spiracles of both the flea and the pupa is remarkably similar. A crucial difference between the two respiratory cycles is the flutter period of the pupa. This cannot stem simply from differences in the intensity of metabolism, for when fleas are placed at temperatures as low as 4° C., which reduces their metabolism to levels characteristic of diapausing pupae, they do not show flutters. Similarly, raising the metabolism of pupae to that of fleas by injury or by other means fails to produce the flea pattern of pro-

longed openings. The reason for the different modes of spiracular behavior is probably to be found, at least in part, in the size of the insects and the attendant differences in the lengths of both fluid and gaseous diffusion paths. Contributing also to the differences in spiracular behavior is the relative insensitivity of the O_2- and CO_2-sensitive mechanisms. This was recognized at the outset by Wigglesworth (personal communication), who pointed out that among the preconditions for discontinuous respiration was that the responding system "must have an extremely high threshold of stimulation by carbon dioxide . . . and this means that there must be a very large accumulation of CO_2 before it will cause the spiracles to open." This is surely so, and the CO_2 threshold of the flea is likely much lower than that of the pupa.

However, the differences between the spiracular behavior of the pupa and the flea are modest when weighed against the similarities. In summarizing his findings on the flea, Wigglesworth (1935) concluded (p. 402) that each respiratory act was "determined by an immediate stimulus of a chemical nature." The present experiments extend these conclusions to the silkworm and indicate that carbon dioxide bursts result in large measure from an exaggeration of a basically simple sort of spiracular behavior.

10. *Comparison with other insects*

When the spiracles of other insect groups are examined, it becomes immediately clear that fluttering, although not unique to silkworms, may be less common than the simple closing and opening observed in the flea. For example, Hazelhoff (1926a) reported no fluttering in any of the Diptera, Trichoptera, Neuroptera and Odonata that he examined. However, he says that in *Periplaneta americana* ". . . All the stigmata are almost closed. . . . The movable stigmal valve performs *usually quick vibrating movements*" (flutters) "whereby nevertheless the opening width of the stigmata remains generally quite small" (p. 70). He adds that ". . . the stigmata in pure O_2 are more closed than in ordinary air," which suggests that here, as in the silkworm, fluttering is controlled by P_{O_2}. Fluttering also occurs in locusts (Hoyle, 1959). How widespread fluttering is in other orders and whether it is usually controlled by P_{O_2} remains to be seen.

11. *Prospects*

The main function of spiracular closing mechanisms is water conservation. From an evolutionary viewpoint, we may imagine that natural selection has favored the development of mechanisms that keep spiracles open just enough to permit the exchange of respiratory gases but otherwise restrict their aperture. Such a water-conserving mechanism might be expected to be best developed in insects with severe water problems, like diapausing pupae which live for long periods without imbibing (*cf.* Imms, 1957, p. 145). As Buck points out (1957, p. 77) discontinuous respiration is an example of such a well-developed spiracular mechanism. And, bursts of carbon dioxide are easily understandable in terms of the spiracular movements, which have been analyzed in earlier sections of this discussion. But what of the disparate rates of gas exchange between bursts, which are the real enigma of discontinuous respiration? Somehow during the interburst the spiracles manage to let oxygen enter, yet simultaneously prevent carbon dioxide

and water vapor from leaving. Whilst the present experiments have not penetrated this secret, they nonetheless provide an experimental framework upon which a theory of mechanism must be based. For the heart of the problem surely lies in the flutter period, where hour after hour the insect practices the "trick" of filtering in oxygen, while retaining carbon dioxide and water. It is our belief that the real significance of the flutter period may have been overlooked in previous explanations of this peculiar one-way transfer of gases. The importance of the flutters in the kinetics of gas exchange will be discussed in a succeeding communication.

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Summary

1. Experiments were conducted to examine the role of the spiracles in discontinuous respiration, to define the kinds of behavior that spiracles can show, and to clarify the manner in which tracheal P_{O_2} and P_{CO_2} interact to provoke various modes of spiracular activity. To accomplish this, records were made of the movements of the spiracular valves of diapausing pupae and developing adults of the Cecropia, Polyphemus and Cynthia silkworms, in air and in gas mixtures.

2. Spiracular valve movements in these silkworms occur in repeated cycles, with periods of from a few minutes to many hours. Each cycle consists of an open period or spiracular burst (which corresponds to the CO_2 burst), a closed or constriction period, and a flutter period which ordinarily occupies most of the cycle. In pupae with long cycles, the respiratory events occur virtually in slow motion when compared with other insects, and this permits careful analysis of complex events in gas exchange and spiracular behavior which are not readily separable in other insects. Evidence is presented that each spiracular act (fluttering, burst, valve closure) is a response to a specific chemical stimulus: the gaseous composition of the tracheal system.

3. In pure O_2, the flutter period is ordinarily eliminated. As ambient P_{O_2} decreases, fluttering reappears and the flutter period progressively lengthens, until, in P_{O_2}'s below 15%, the spiracular bursts disappear and fluttering is continuous.

4. Ambient P_{CO_2} ordinarily has no effect until it increases above 5%, whereupon the cycles shorten. This shortening occurs at the expense of the flutter period, which progressively diminishes as P_{CO_2} increases. When P_{CO_2} rises above about 15%, the cycles break down completely and the valves flutter continuously.

5. Intubating even one pupal spiracle eliminates the cycles in the remaining 13, and the valves stay permanently constricted, presumably because normal triggering stimuli for spiracular activity are absent.

6. The spiracles of intubated pupae can be caused to open by lowering the P_{O_2} or raising the P_{CO_2}. The P_{CO_2} which opens the spiracles varies with the ambient P_{O_2}: in a typical pupa, in 2.3% O_2, 5% CO_2 opened the spiracles, whereas in 4.7% O_2, 10% CO_2 was required.

7. From these and other data, it is concluded that the cyclical movements of the spiracles result from cyclical changes in tracheal composition. In particular, fluttering is initiated by low P_{O_2}, while spiracular bursts are caused by high P_{CO_2}.

8. Evidence is presented to prove that the pupa possesses an independent O_2-sensitive mechanism, which is quite separate from any CO_2-sensitive mechanism. It is also argued that low P_{O_2} does not affect spiracular behavior by virtue of anoxia-produced acidity, nor by virtue of acidity releasing bound carbon dioxide.

9. The spiracular behavior of these silkworms is compared in detail with the picture of spiracular behavior in the flea provided by Wigglesworth, and it is concluded that there is no fundamental difference between the two, except for the flutter period which seems peculiar to the pupa. Evidence is presented that the flutter period holds the key to the disparate rates of gas exchange between bursts, which remains the central problem of discontinuous respiration.

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FURTHER STUDIES ON THE TREMATODE GENUS HIMASTHLA WITH DESCRIPTIONS OF H. MCINTOSHI N. SP., H. PISCICOLA N. SP., AND STAGES IN THE LIFE-HISTORY OF H. COMPACTA N. SP.

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The genus *Himasthla* is a member of the Echinostomatidae, a family which comprises a large number of genera and species that live in the digestive tracts of birds and mammals. Dietz (1909) erected the genus with *H. rhigedana,* a new species from the curlews, *Numenius arquatus* and *Numenius arabicus,* as type. He (1910) characterized the family and the several genera. As members of *Himasthla* he included *H. militaris* (Rudolphi, 1803) from the European curlew, *Scolopax* (= *Numenius*) *arquata; H. leptosoma* (Creplin, 1829) from *Tringa alpina* (= *variabilis*); *H. elongata* (Mehlis, 1831) from species of gulls, *Larus; H. secunda* (Nicoll, 1906) from the black-headed gull, *Larus ridibundus* and the herring gull, *Larus argentatus;* and *H. alincia* Dietz, 1909, based on specimens from the semipalmated sandpiper, *Tringa pusillus* collected by Natterer in Brazil. The first of these species was taken on the Sinai peninsula, the last in South America, while the others were European. As important criteria for specific determination, Dietz listed the number of spines on the collar, the extent of the vitellaria, and the structural details of the copulatory organs.

Subsequently described species include *H. harrisoni* Johnston, 1917 from *Numenius cyanopus* in Australia; *H. incisa* Linton, 1928 from the white-winged scoter, *Oidemia deglandi,* at Woods Hole, Massachusetts; *H. muehlensi* Vogel, 1933 from a human patient in Hamburg, Germany; *H. ambigua* Palombi, 1934, based on encysted metacercariae from the gills of *Tapes decussatus* in the Mediterranean; *H. kusasigi* Yamaguti, 1939 from *Tringa ochlopus* and *H. megacotyle* Yamaguti, 1939 from *Erolia alpina sakhalina,* both species taken in Japan; *H. multilecithosa* Mendheim, 1940 from a captive great crowned pigeon, *Goura coronata;* and *H. tensa* Linton, 1940 reported from a codfish, *Gadus morrhua,* at Woods Hole, Massachusetts. Stunkard (1934, 1937, 1938) showed that *Cercaria quissetensis* Miller and Northup, 1926 is the larval stage of a species of *Himasthla,* for which the larval name was adopted. The asexual generations occur in *Nassarius obsoletus,* the cercariae penetrate and encyst in different species of mollusks, and the worms become sexually mature in the intestine of gulls. Prudhoe (1944) described, but did not name, a single specimen from the yellow-wattled lapwing, *Lobipluvia malabarica,* and assigned it provisionally to the genus *Himasthla.*

Metacercariae from the foot of *Scrobicularia tenuis* were identified as *Cercaria leptosoma* by Villot (1878), who traced their development to maturity in the

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alimentary tract of the "allouette de mer à collier," *Tringa variabilis*. The number of collar-spines was not recorded, but the figure of the adult is very similar to that of *H. leptosoma* as given by Dietz. Cuénot (1892) reported metacercariae of *H. leptosoma* encysted in the circumoral tentacles of *Synapta inhaerens* and in the foot of *S. tenuis*, but since he found 31 or 32 collar-spines, it is obvious that he was dealing with some species other than *H. leptosoma*. Palombi (1925) identified metacercariae from *Mytilus galloprovincialis* as larvae of *Echinostomum secundum* (= *H. secunda*) Nicoll, 1906. Skrjabin (1956) included figures of the larval stages of *H. militaris* after the work of Zelikman, but there was no account of the life-cycle or reference to other published report.

There has been much difference of opinion concerning specific distinctions and the number of valid species in *Himasthla*. Stunkard (1939) reviewed the history of the genus and discussed the problem of specific determination. Linton (1928) had described specimens from four species of gulls (*Larus*) and others from the white-winged scoter, *Oidemia deglandi*, and from the black-crowned night heron, *Nycticorax nycticorax*. The worms from *O. deglandi* were described as a new species, *H. incisa*, while all the others were referred to *H. elongata*, despite differences in number of collar-spines and other morphological features. Some of the worms had 29, others 31 collar-spines, and it was clear that two distinct species were represented. Those with 31 spines were identified by Stunkard (1938) as *H. quissetensis*, whereas those with 29 spines were intermediate in size and morphology or overlapped the figures given by Dietz (1910) as characteristic for *H. elongata* and *H. militaris*. Stunkard (1938) stated, p. 190, "In my opinion the worms might with equal justification be referred to either of the two species. On the other hand, they may belong to neither." Sprehn (1932) listed *H. militaris* and *H. secunda* as synonyms of *H. leptosoma* and Palombi (1934) admitted the probable identity of *H. leptosoma* and *H. secunda*. Stunkard noted similarity between *H. alincia* and *H. quissetensis* and conceded their possible identity. Dawes (1946) regarded *H. leptosoma* and *H. elongata* as valid species, but listed *H. militaris* and *H. secunda* as synonyms of *H. leptosoma*.

In correspondence with Dr. Vogel, Dr. Mendheim accepted the opinion of Sprehn (1932) and in addition predicated the identity of *H. elongata* and *H. leptosoma*. With this proposal, all the specimens of *Himasthla* with 29 collar-spines were included in a single species, *H. elongata*. However, if the idea were correct, the name of the species must be *H. militaris* (Rudolphi, 1803), which has priority over *H. elongata* (Mehlis, 1831). In addition, Dr. Mendheim suggested the identity of *H. muehlensi* and *H. elongata*. The specimens of *H. muehlensi* had been recovered from the stool of a patient following a purgative after his arrival at the hospital of the Institut für Schiffs- und Tropenkrankheiten in Hamburg, Germany. The patient was a South American who had stopped at New York on his way to Germany and in sea-food restaurants had eaten many "clams," juvenile *Venus* (*Mercenaria*) *mercenaria* which are served raw on the half-shell under the designation "cherry stones." In his description of the specimens, Vogel (1933) noted that members of *Himasthla* are typically parasites of birds and that the human infection was a recent and probably an accidental one. In a monographic study of the Echinostomidae, Mendheim (1940) described *Himasthla multilecithosa* n. sp., from *Goura coronata*, a native of the Papuan and Solomon Islands. Mendheim reported

that the time and place of the infection were unknown, that the normal food of these birds is fruit, and since the infective larvae of *Himasthla* occur in marine mollusks, the infection must have been accidental or incidental. After redescriptions of *H. elongata* and *H. muehlensi* he wrote, p. 578, "Auf Grund einiger Befunde müssen *Himasthla leptosoma, Himasthla elongata* und *Himasthla muehlensi* zu einer Art vereinigt werden." But his account of *H. elongata* was obviously based on material of more than one species since he reported the number of collar-spines as 29–31. His report on the specimens of *H. muehlensi,* loaned by Dr. Vogel, adds nothing of significance to the original description. He noted that egg production had just begun and that the first eggs were smaller than those formed later, from which he concluded, p. 511, "Die Eimasse können also nicht als brauchbares Artkriterium gelten." The observation is not new (q.v., Beaver, 1937, p. 26) and the conclusion is equivocal.

The writer had the opportunity in Hamburg to study the specimens of *H. muehlensi,* several specimens of *H. leptosoma* from *Tringa alpina* in the collection of Dr. Vogel, and four specimens sent by Dr. Mendheim that were identified as *H. elongata.* In a report, Stunkard (1939) stated that the specimens of *H. muehlensi* have 31 collar-spines and while there is a possibility that they may be identical with *H. quissetensis,* they certainly are distinct from *H. elongata* which has 29 spines. Furthermore, although *H. elongata* and *H. leptosoma* both have 29 collar-spines, morphological differences, first listed by Dietz and manifested by specimens from the collections of Vogel and Mendheim, clearly differentiate the two species. Specimens of *H. leptosoma* are smaller, the structure is more delicate, the suckers, collar-spines and eggs are smaller, the vitellaria do not extend as far anteriad, and the gonads are larger. Mendheim (1943) reaffirmed the identity of *H. elongata, H. leptosoma* and *H. muehlensi,* which he derived from average measurements of 15 specimens which, according to the text, included the four worms identified by Stunkard as *H. elongata.* Since the author admittedly was unable to distinguish between *H. elongata* and *H. leptosoma,* and since it is apparent from his figures that the 15 specimens included representatives of both species, the average measurements are meaningless. In a key for determination of species, Mendheim (1943, p. 235) distinguished *H. incisa* on the basis of 27 collar-spines. In the original description Linton (1928) wrote, p. 12, "The exact number of oral spines could not be made out in the balsam mount. There are at least 27. There is a single row, except at the lateral angles." After examination of the type specimen, Stunkard (1938) reported that the worm has 31 spines. Re-examination of the specimen confirms that number; the spines are relatively stout and those in the single row measure 0.065 to 0.070 mm. in length. The slide bearing the single type specimen has a label written by Professor Linton noting that the worm was collected by Vinal Edwards, June 2, 1914, at Woods Hole, Massachusetts.

Linton (1940) described *H. tensa* as a parasite of *Gadus morrhua;* there are three specimens on the slide deposited in the U. S. National Museum and the notation, in Linton's handwriting, states that the worms were collected by Vinal Edwards, 1915. Mendheim apparently accepted the fish as a normal host. He stated, (1943, p. 235) "Besonders bemerkungswert ist *H. tensa* aus *Gadus morrhua.* Es ist dies seit dem von Diesing beschriebenen *Echinostoma annulatum* Diesing 1850 die einzige Echinostomiden-Art aus Fischen. Körperform, Lage des Bauch-

PLATE I

FIGURE 1. *Himasthla compacta,* type specimen; length 4.00 mm.

FIGURE 2. *Himasthla compacta,* cotype specimen; length 3.10 mm.

FIGURE 3. *Himasthla elongata,* juvenile specimen without eggs, from *Larus delawarensis,* taken 24 January 1914 at Woods Hole, Mass., by Vinal Edwards. Linton material now in U. S. National Museum Helminthological Collection, No. 7922; length 6.00 mm.

FIGURE 4. *Himasthla elongata,* from *Larus argentatus,* taken 18 December 1921 at Woods Hole, Mass., by R. A. Goffin. Linton material now in U. S. National Museum Helminthological Collection, No. 7921; specimen 8.75 mm. long.

FIGURE 5. *Himasthla elongata,* on same slide as Figure 4, same data; specimen 6.75 mm. long.

FIGURE 6. *Himasthla elongata prov.,* from night heron, *Nycticorax nycticorax,* taken 11 September 1914 at Woods Hole, Mass., by Vinal Edwards. Linton material now in U. S. National Museum, No. 7924; specimen 6.00 mm. long.

PLATE II

FIGURE 7. *Himasthla tensa* Linton, 1940. Type and cotype specimens from *Gadus morrhua*, taken 22 January 1915 at Woods Hole, Mass., by Vinal Edwards. Linton material now in the U. S. National Museum, No. 8214; type specimen at bottom of photograph; length, 5.32 mm.

saugnapfes und Pseudosegmentierung lassen es vor allem seit Linton's Fund als durchaus wahrscheinlich erscheinen, dass Diesing eine *Himasthla*-Art vorgelegen hat." *Distomum annulatum* was described by Diesing (1850) from the intestine of the electric eel, *Gymnotus electricus*. The specimens had been collected by Natterer in Brazil, the 3rd of September, 1827. In his description and figures, Diesing (1855) gave the length as 6 lines and the maximum width as ½ line, which would be slightly more than 12 by 1 mm. Cobbold (1860) transferred the species to *Echinostoma* and Dietz (1910) listed it under *Species inquirendae*. Mendheim noted features in which it conforms to the diagnosis of *Himasthla*. The specific name, *annulatum*, is significant since this feature is a prominent characteristic of the genus. Examination of the figures of Diesing (1855) and comparison

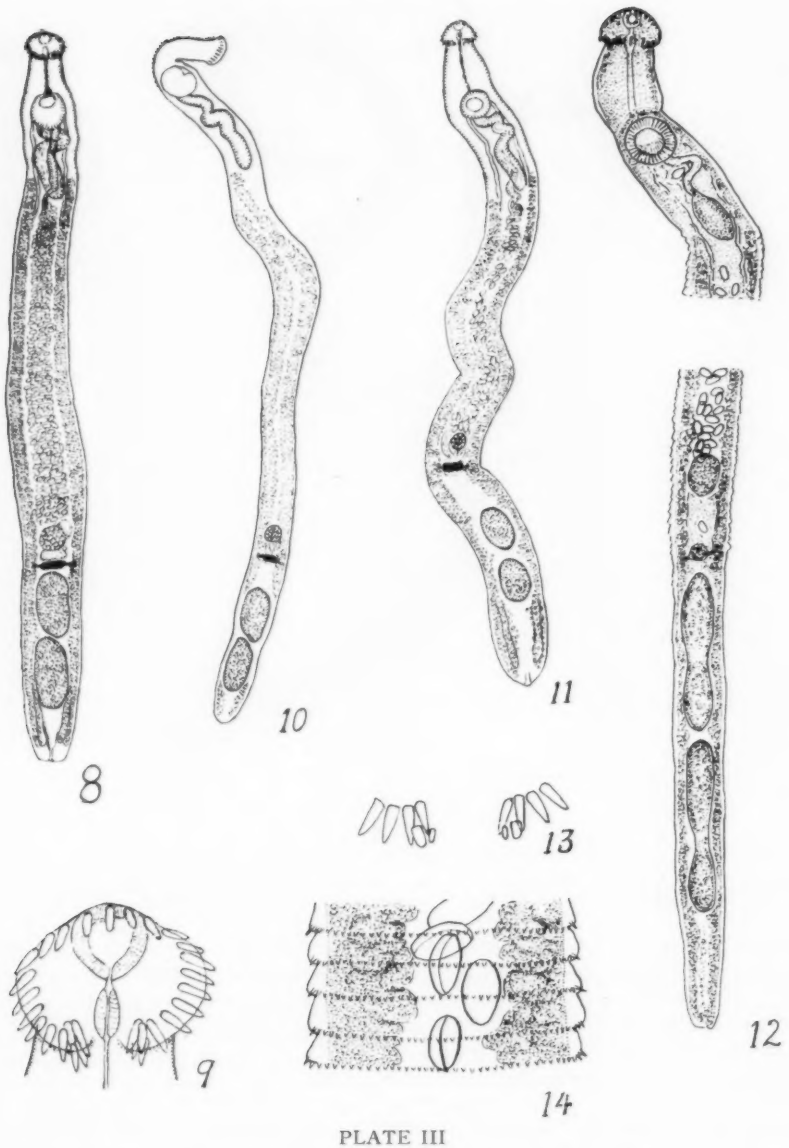

PLATE III

FIGURE 8. *Himasthla elongata*, drawing of specimen shown in Figure 5.
FIGURE 9. Collar and spines of specimen shown in Figure 8.
FIGURE 10. *Himasthla elongata*, drawing of specimen shown in Figure 3.

of his Figure 21, Plate III, showing five annuli, with Figure 14 of the present work, show that the worms described as *D. annulatum* agree far better with the generic concept of *Himasthla* than with that of *Echinostoma*. Accordingly they are transferred to that genus as *Himasthla annulata* (Diesing, 1850). Although *H. annulata* and *H. tensa* were described from fishes, each species has been found only once and there are strong reasons for regarding both of them as parasites of birds which were found incidentally in the intestine of the electric eel in Brazil and the codfish of New England, respectively.

Material and Methods

This report presents results from one section of the investigation conducted by the U. S. Bureau of Commercial Fisheries to discover causes for the decline in number of soft-shelled clams, *Mya arenaria*, along the coast of New England. To Walter R. Welch, Chief, Clam Investigations, and his staff in Boothbay Harbor, Maine, grateful appreciation is expressed for avian and molluscan specimens provided for the study. Other material was provided by the Marine Biological Laboratory, Woods Hole, Massachusetts, where the experimental work was done. Examination of clams from the region of Boothbay Harbor disclosed echinostome metacercariae encysted in the palps and gills. These larvae were fed over periods of one to seven weeks to laboratory-reared eider ducks (*Somateria mollissima*), herring gulls (*Larus argentatus*), terns (*Sterna hirundo*), white mice and golden hamsters. The worms excysted in eider ducks but did not persist more than about ten days. Adult worms were recovered from only one species, *L. argentatus*. Further study has shown that three species, all members of the genus *Himasthla*, are concerned. One of these species, *H. quissetensis*, was described by Stunkard who (1938) found the asexual generations in the mud-snail, *Nassarius obsoletus;* the life-history of another, described as *H. compacta* n. sp., is reported in the present paper; while the third species is identified as *H. elongata* (Mehlis, 1831). All three species have been recovered from the intestine of laboratory-reared gulls, *L. argentatus*, fed palps and gills of *M. arenaria* collected in the Boothbay Harbor area. To preclude accidental and complicating infections, the birds were fed commercial, canned cat-food, made from fishes and supplemented with vitamins. The examination of shore-birds in the Boothbay Harbor area disclosed a fourth species, identified as *H. alincia*, in the intestine of *Ereunetes* (*Tringa*) *pusilla*. This is the host from which the species was originally described by Dietz (1909).

To supplement the study of these worms, all specimens of *Himasthla* in the Helminthological Collection of the U. S. National Museum have been received on loan through the kindness of Dr. Allen McIntosh and the favor is here gratefully acknowledged. The collection contains several specimens from *Larus argentatus*

Figure 11. *Himasthla elongata*, drawing of specimen shown in Figure 6.

Figure 12. *Himasthla elongata*, a greatly extended specimen from the intestine of *Larus argentatus*, experimental infection; the bird had been fed metacercariae encysted in the gills and palps of *M. arenaria*. In the drawing, a section 6 mm. long, containing 110 annuli, is omitted from the middle of the body; specimen 12.5 mm. long.

Figure 13. Spines at the ventro-lateral corners of the collar; note the very small median spines.

Figure 14. Five annuli from the central portion of the specimen shown in Figure 12.

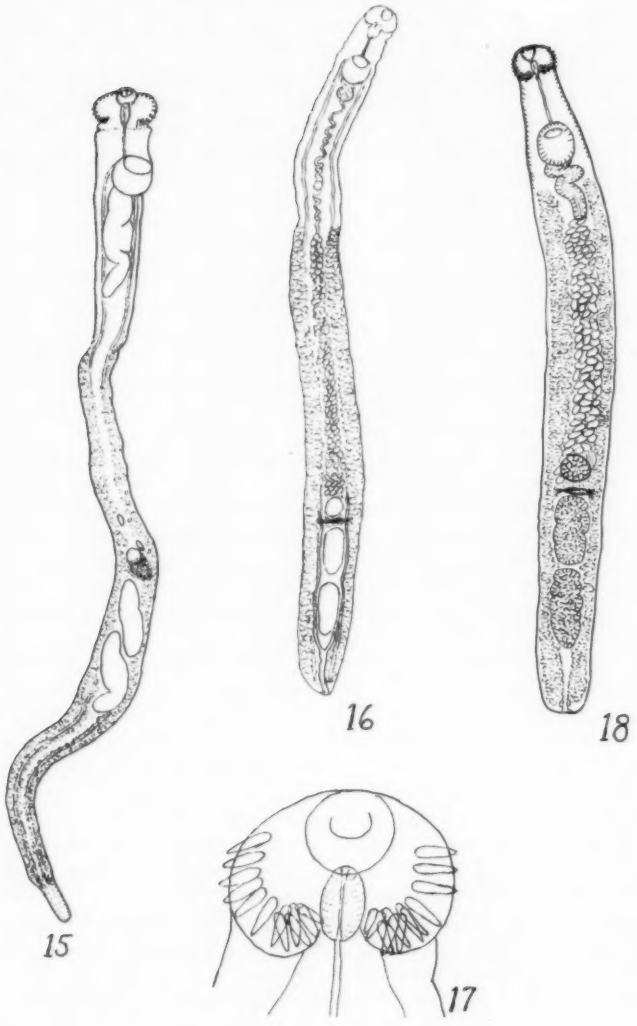

PLATE IV

FIGURE 15. *Himasthla piscicola,* type specimen, length 9.80 mm.
FIGURE 16. *Himasthla mcintoshi,* type specimen, length 8.00 mm.
FIGURE 17. Spines at the ventro-lateral corners of the collar of worm shown in Figure 16.
FIGURE 18. *Himasthla compacta,* drawing of specimen shown in Figure 1.

and one from *Nycticorax nycticorax* which had been identified by Linton as *H. elongata*. It contained, also, the type material of *H. incisa* Linton, 1928 and *H. tensa* Linton, 1940. Examination of the specimens of *H. tensa* discloses that the number of collar-spines is 29, not "about 32" as reported by Linton (1940). In the three specimens (Fig. 7), the vitelline follicles terminate a short distance posterior to the caudal end of the cirrus-sac. In the specimens of *H. elongata* shown in Figures 4 and 5, the vitellaria extend anteriad slightly past the caudal end of the cirrus-sac but in other individuals, especially younger ones, the vitellaria do not extend to the level of the cirrus-sac. No constant differences were found between these worms and, accordingly, the name *H. tensa* is suppressed as a synonym of *H. elongata*. The Museum Collection also contains four slides bearing the number 54,721, with seven specimens from the "long-billed curlew," *Numenius americanus americanus*, collected by J. Bushman at Orr's Ranch, Tooele Co., Utah, April 21, 1954. These worms have 35 collar-spines and are described as a new species, *Himasthla mcintoshi*. Subsequently, Dr. George R. La Rue of the Research Center, U. S. Department of Agriculture, Beltsville, Maryland, sent two specimens taken from the digestive tract of a South American fish, *Arapaima gigas*, that died in the Toledo, Ohio, zoo and that had been referred to him for identification. Grateful acknowledgment is made also to Dr. La Rue for the privilege of examining and describing these worms.

To locate the asexual generations of the metacercariae which occur in the gills and palps of *M. arenaria*, a survey of the mollusks in the Boothbay Harbor area was begun. As noted above, *N. obsoletus* had been identified as the first intermediate host of *H. quissetensis*. The first intermediate hosts of *H. elongata* and *H. alincia* are yet unknown, but there is strong and almost conclusive evidence that an echinostome cercaria from *Hydrobia minuta* is the larval stage of one of the metacercariae in *M. arenaria* and that this metacercaria develops in *L. argentatus* to adults described in this paper as *Himasthla compacta* n. sp. *Hydrobia minuta* harbors at least six different species of cercariae. Examination of 5000 snails, isolated 100 per dish, yielded three specimens shedding an echinostome cercaria and examination of 5000 snails in another series, in which an individual count was kept as the snails were crushed and examined under the microscope, disclosed six infections by this echinostome cercaria. It has 29 collar-spines and is the only echinostome cercaria, other than that of *H. quissetensis* which has 31 spines, found so far in the region. Very small specimens of *M. arenaria* collected in the Woods Hole, Massachusetts, area were added to the dishes containing the cercariae from *H. minuta* and metacercariae, presumably of experimental infection, were recovered from them. Owing to the low incidence of infection and the small size of the snails, and the resulting scarcity of cercariae, it has not been possible to produce massive infections which are easily obtained with the abundant cercariae of *H. quissetensis*. When using clams collected in the field, there is the possibility that they may be carrying metacercariae of natural infection, but examination of 200 specimens from the area where those used in the experiment were taken, revealed no infection. Since laboratory-reared clams were not available, very small clams from the Woods Hole area were employed. These clams, exposed to the cercariae, were fed subsequently to a young, laboratory-reared gull, *L. argentatus*. The bird began to pass trematode eggs some four weeks after the first feeding and later, on examination, it contained 26 small

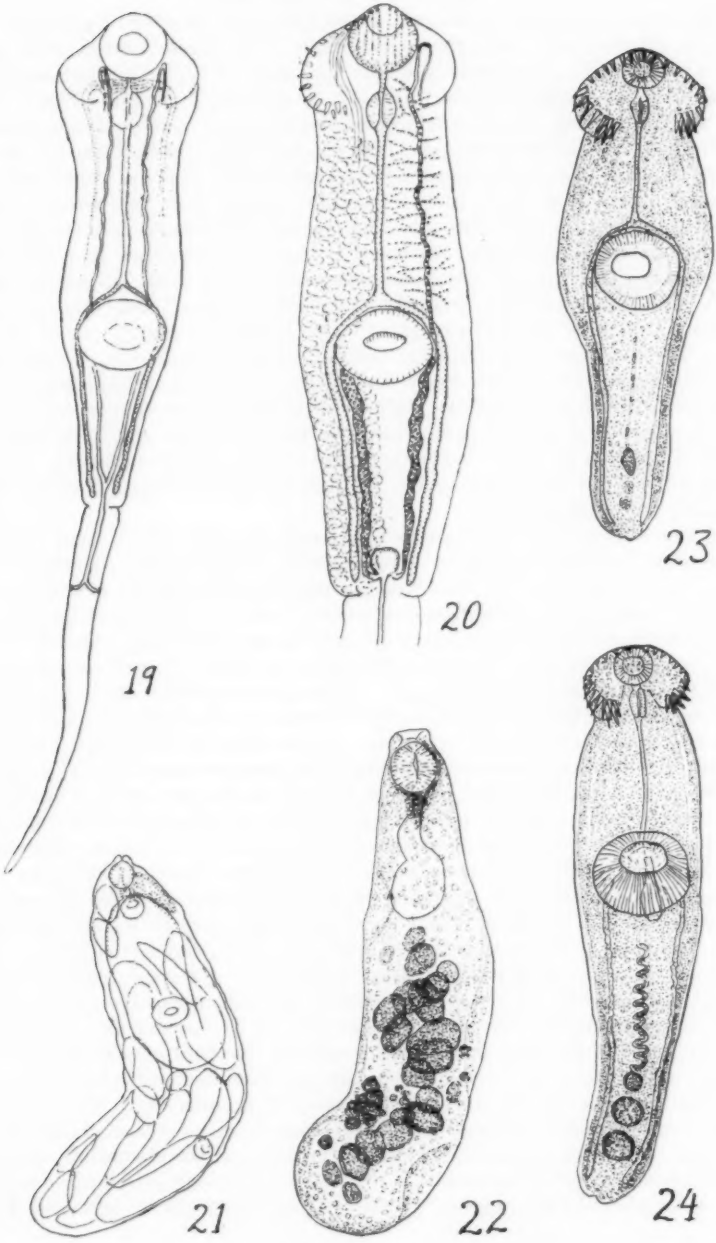

PLATE V.

echinostomes, identified as *H. compacta* n. sp. This species has 29 collar-spines and identification of the larval stages is not easy. The sizes of the cercariae and of their metacercariae are not significantly different from those of *H. quissetensis* and do not provide certain criteria for specific determination of the larvae. The adults are distinguished easily. Consequently, it appears that one of the best means of positive identification is to expose small clams to the cercariae and then feed the metacercariae to previously unexposed birds.

Additional data on the life-cycle are provided by results of attempts to infect the snail host. Eggs of *H. compacta*, taken from worms that had developed in a laboratory-reared gull, were incubated in sea-water at room temperature. At the end of four weeks they contained fully formed miracidia and were added to a finger-bowl containing 20 juvenile *H. minuta*. Four weeks later the number of living snails was reduced to 13. Snails that died were not examined, since the tissues decomposed quickly and trematode larvae would probably not be recognizable. One snail, dissected four weeks after exposure, contained three small rediae (Fig. 22) but whether they were mother or daughter rediae was not determined. No cercariae were obtained and the experiment was disappointing; however, it illustrates the difficulties inherent in this type of research. The number of eggs was limited, development seemed to proceed at variable and inconstant rates, and actual hatching was not observed. Since individual exposure of a snail to one or more miracidia could not be made, the mass exposure method was undertaken to determine whether or not certain of the miracidia on emergence could find and infect snails. With such "shotgun" technique, it is impossible to know how many, if any, miracidia penetrated a particular snail and it is possible that the death of certain snails was caused by superinfection. The one experimental infection, however, supplements other data and strongly supports the postulated life-cycle.

Descriptions

Himasthla elongata (Mehlis, 1831)

Linton (1928) described trematodes from *Larus argentatus* at Woods Hole, Massachusetts, which he identified as *Himasthla elongata*. Stunkard (1938) pointed out that the material of Linton comprised two distinct species, one of which was identified as *Himasthla quissetensis*, whose cercarial stage had been described

PLATE V

Figure 19. *Himasthla compacta*, young cercaria from *Hydrobia minuta*, natural infection, specimen fixed and stained; body 0.50 mm. long; tail 0.37 mm. long; to show digestive, nervous and excretory structures.

Figure 20. *Himasthla compacta*, normally emerged cercaria, sketch of living specimen to show glands and excretory ducts.

Figure 21. *Himasthla compacta*, redia, natural infection, fixed and stained; specimen 1.12 mm. long.

Figure 22. *Himasthla compacta*, redia, experimental infection, fixed and stained; specimen 0.47 mm. long.

Figure 23. *Himasthla compacta*, young specimen from gull, fixed and stained; 0.46 mm. long.

Figure 24. *Himasthla compacta*, young specimen from gull, fixed and stained; 0.76 mm. long.

and named by Miller and Northup (1926). The members of the other species were not identified although Stunkard noted that measurements of 12 representative specimens are intermediate between or overlap the figures given by Dietz (1910) as characteristic for *H. elongata* and *H. militaris*.

Certain of the adult worms recovered from the intestine of *L. argentatus* after feeding metacercariae encysted in the tissues of *M. arenaria* from Maine are identified as *H. elongata*. These worms unquestionably are specifically identical with those identified as *H. elongata* by Linton (1928). In most of the specimens the collar-spines are slightly larger and the testes considerably larger than the figures given by Dietz (1910) for *H. elongata* but there is considerable variation, and the location of the testes and ovary near the posterior end of the body clearly differentiates them from *H. militaris*. One specimen, killed in a very extended condition (Figs. 12, 13, 14), exhibits the pseudosegmentation characteristic of the postacetabular region of the body and the annular arrangement of the flattened, scale-like spines. In this specimen, the two median members of the corner-spines (Fig. 13) on the collar are very small, recalling the condition shown in Figure B^1 of Dietz. The worm measures 12.5 mm. in length and 0.45 mm. in greatest width. In Figure 12, 6 mm. and 110 annulations are omitted from the middle of the body. Five of the annuli are portrayed in Figure 14. Another, younger specimen, with a few eggs in the uterus gave the following measurements: length, 4.4 mm.; width, 0.5 mm.; width at collar, 0.3 mm.; spines, 0.058 mm. long; acetabulum, 0.31 mm. long and 0.26 mm. wide; oral sucker, diameter 0.11 mm.; pharynx, 0.098 mm. long and 0.056 mm. wide; vitellaria do not extend forward to the level of the cirrus-sac; ovary 0.156 mm. wide and 0.125 mm. long; anterior testis, 0.25 mm. long and 0.155 mm. wide; posterior testis, 0.28 mm. long and 0.15 mm. wide; eggs 0.090 to 0.100 mm. long and 0.060 to 0.068 mm. wide. Gravid worms vary in size from these measurements to older individuals that are twice as large with correspondingly larger organs, but the structural pattern of the species is always evident.

The single specimen from the night heron, *N. nycticorax*, shown in Figures 6 and 11, has 29 collar-spines but manifests certain differences from the worms found in *L. argentatus*. It is only slightly smaller than the worm shown in Figure 5; however, the collar-spines and suckers are smaller, the vitellaria do not extend as far anteriad, and the gonads are smaller and slightly farther forward. In this specimen the spines are 0.050 mm. long; the oral sucker measures 0.114 by 0.107 mm.; the acetabulum is 0.325 mm. long and 0.290 mm. wide; the ovary is spherical, 0.143 mm. in diameter; the anterior testis is 0.40 mm. long and 0.23 mm. wide; the posterior testis is 0.42 mm. long and 0.25 mm. wide; the eggs average 0.097 by 0.062 mm. The measurements are almost identical with those given by Nicoll for *Himasthla secunda*, and the small size of the gonads agrees well with the description of *H. elongata* as given by Dietz. The similarity suggests possible identity of *H. secunda* and *H. elongata*. Since the material at hand consists of a single specimen, it is referred for the present to *H. elongata*. When the life-history of that species is known and cercariae are available for experimental infections, it will be possible to determine definitely whether the present worm belongs in *H. elongata* or is a member of some other species.

The specimen from *Larus delawarensis* (Fig. 3) is referred to *H. elongata*, but it is juvenile and possibly members of that species do not attain sexual maturity in *L. delawarensis*.

Himasthla compacta n. sp.

Adult

Material of this species consists of worms recovered from laboratory-reared *L. argentatus* fed *M. arenaria* collected near Boothbay Harbor, Maine, and others from another laboratory-reared *L. argentatus,* five weeks after the beginning of an experiment in which the bird was fed metacercariae encysted in small *M. arenaria.* These clams had been exposed to echinostome cercariae from *H. minuta* collected in Sagadahoc Bay, near Boothbay Harbor, Maine, and the metacercariae were presumed to be encysted stages of the same cercariae. Most of the worms were sexually mature but several were juveniles. The bird had been fed five weeks, two weeks and one week before it was killed and the small worms shown in Figures 23 and 24 are probably from the last two feedings. A specimen 2.130 mm. long and 0.275 mm. wide does not have eggs in the uterus, although there are spermatozoa in the testes.

Gravid specimens, fixed and stained, measure 3.00 to 4.30 mm. in length and 0.35 to 0.44 mm. in width. For such a small species of *Himasthla,* the organs are large and compactly disposed. The sides of the body are almost parallel; the acetabulum protrudes; in the preacetabular region the lateral edges are often turned ventrad and mediad, forming a ventral depression. The anterior end has a reniform collar, open ventrally, which bears 29 spines, 25 arranged in a linear row and two smaller corner-spines on each side behind the terminal ones of the row. The lineal spines are 0.054 to 0.062 mm. in length and 0.012 to 0.014 mm. wide; the smaller corner ones are 0.026 to 0.032 mm. long and 0.009 mm. wide. In mature specimens the acetabulum is about one-seventh of the body length from the anterior end whereas in juvenile worms it is relatively farther back and in young worms it is near the middle of the body. The shift in relative position of the acetabulum results from development of the reproductive organs in the postacetabular portion of the body. The acetabulum is usually longer than broad, oriented with the opening at the antero-ventral face, just behind the common genital pore. The sucker measures 0.20 to 0.26 mm. in length and 0.18 to 0.22 mm. in width. The cuticula in the preacetabular area bears scale-like spines arranged in an imbricate pattern, while behind the sucker the spines are smaller and are arranged in the annular fashion characteristic of the genus.

The mouth is subterminal; the oral sucker measures 0.075 to 0.090 mm. in diameter. There is a short prepharynx; the pharynx is oval to pyriform, usually wider posteriorly, it measures 0.060 to 0.075 mm. in length and 0.040 to 0.050 mm. in width. The esophagus extends almost to the acetabulum and the ceca end blindly near the posterior end of the body.

The testes are almost contiguous, one behind the other, in the caudal one-third of the body. They are oval, with notched but not lobed surfaces. The posterior testis is usually somewhat larger than the anterior one. The anterior testis is 0.36 to 0.49 mm. in length and 0.18 to 0.21 mm. in width; the posterior testis is 0.40 to 0.58 mm. in length and 0.18 to 0.21 mm. in width. From each testis a sperm duct passes forward and the two unite just before reaching the cirrus-sac. The common duct, on entering the sac, expands into a coiled seminal vesicle, which fills the posterior one-half or more of the cirrus-sac. The vesicle is followed by a shorter prostatic portion of the duct and then by a protrusible cirrus armed with very small

spines. The cirrus-sac extends behind the acetabulum more than the diameter of that sucker and terminates between the anterior ends of the vitelline glands.

The ovary is spherical to oval, usually broader than long, situated from two-sevenths to three-eighths of the body length from the posterior end. It is about its diameter in front of the anterior testis. It measures 0.06 to 0.13 mm. in length and 0.08 to 0.16 mm. in width. The oviduct arises at the posterior face and passes backward where it enters the ootype region. It expands somewhat and gives off Laurer's canal, which passes to the dorsal surface of the body, after which it receives the common vitelline duct. This portion is partially enclosed in the cells of Mehlis' gland, which surrounds the ootype. The initial portion of the uterus is filled with masses of spermatozoa. The uterus coils about and passes forward to the level of the caudal end of the cirrus-sac where it joins the metraterm. Both metraterm and cirrus-sac pass forward above the acetabulum to open into a shallow genital sinus, and the genital pore is on the median ventral surface, immediately anterior to the acetabulum. The vitelline follicles are lateral to and partially overlap the digestive ceca; they extend from the caudal end of the body to a level about the length of the acetabulum behind that sucker. They are not interrupted at the levels of the testes. Longitudinal ducts connect the follicles and transverse ducts pass mediad at the level of the ootype, where they unite to form a vitelline receptacle from which a common duct leads to the initial portion of the ootype. The eggs are large, oval, thin-shelled, collapsed in the preserved specimens, 0.085 to 0.090 mm. in length and 0.050 to 0.058 mm. in width. Each egg contains an ovum and several vitelline cells. The ovum is situated toward the opercular end of the egg. Cleavage begins in the uterus, but development there does not go much beyond the four-cell stage.

Himasthla compacta differs from all other species of the genus in its smaller size, its compact structure and relatively larger gonads. The ovary is situated about one-third of the body length from the posterior end and the testes extend through most of the postovarian distance, a condition not found in any other species. Since *H. compacta* has not been reported previously from *L. argentatus*, some other avian species may be its normal host.

The type specimen (Figs. 1, 18) is deposited in the U. S. National Museum Helminthological Collection under the number 39,444.

Redia

In structure and behavior, the rediae are very similar to those of *H. quissetensis* as described by Stunkard (1938). Figure 21 is a drawing of a large, gravid redia of natural infection. It is 1.12 mm. long and 0.28 mm. wide; the pharynx is 0.028 mm. in diameter. Figure 22 shows a young redia recovered from one of the small specimens of *H. minuta* which had been exposed for four weeks to embryonated eggs of *H. compacta* taken from worms that had developed in the intestine of a laboratory-reared specimen of *L. argentatus*. Whether this is a first or second generation redia could not be determined, since the germ balls in it could be embryos of either rediae or cercariae. At this stage they are quite indistinguishable. The specimen measures, fixed, stained and mounted, 0.47 mm. long, 0.12 mm. wide, and the pharynx is 0.027 mm. in diameter. The young rediae have collars which become visible as the larvae move; they progress in a lumbricid manner, with temporary protrusions of foot-like projections to anchor one region while advancing another. In young rediae

the gut is conspicuous, filled with yellowish-green material, obviously the residue from digestion of snail tissue. The intestine does not increase in size and becomes proportionately smaller as the cavity of the redia becomes filled with larvae of the next generation. With increase in size and accumulation of growing progeny, the rediae become less and less active. Meanwhile, with advancing maturity, the cercariae become more vigorous in their movements until they emerge through the birth-pore, situated near the pharynx.

Cercaria

The incidence of infection of *H. minuta* is low, about one in five hundred, and cercariae emerged from less than one-half of the infected snails. The snails are small and few cercariae are produced. They leave the rediae and complete development in the haemocoele of the snail. In a crushed snail with mature infection, usually there are two to four or five cercariae free in the sinuses and others still in rediae. Some of those in the rediae swim actively when released, but the small number of cercariae restricts experimental procedure. In swimming, the body is contracted until it is almost spherical while the tail is extended and lashes vigorously.

The body in living cercariae measures 0.30 to 0.60 mm. in length and 0.08 to 0.19 mm. in width; it is elongate oval in outline although the shape varies with the degree of elongation and retraction. The collar gives the anterior end a characteristic form and when the larva is fully extended, this may be the widest part of the body. The tail is shorter than the body and capable of great extension and retraction by contraction of the circular or longitudinal muscles which form its wall. In naturally emerged cercariae the oral sucker measures 0.057 to 0.065 mm. in diameter; the ventral sucker 0.072 to 0.086 mm.; and the pharynx 0.014 to 0.020 mm. Figure 19 is of a well extended young cercaria from a crushed snail, fixed with hot AFAG (alcohol-formalin-acetic acid-glycerin) fixing fluid added to a small amount of sea-water in a beaker in which the larvae were being whirled. Stained and mounted, it has a total length of 0.87 mm.; the body is 0.50 mm. long and 0.13 mm. wide at the collar; the acetabulum is 0.080 mm., the oral sucker is 0.057 mm., and the pharynx is 0.020 mm. in diameter. The tail is 0.037 mm. wide at the base. The body of the cercaria contains three types of glandular cells. There are three pairs of penetration glands situated in the preacetabular area, and four pairs of glands in the oral sucker, all of which open through pores at the anterior end of the body. The entire dorsal area of the body is occupied by cystogenous cells whose cytoplasm is filled with bacilliform granules. These granules do not stain with vital dyes. Other glandular cells, more ventral in location, stain faintly with eosin and erythrosin.

The reproductive organs are represented by two groups of deeply staining cells, one near the posterior end of the body and the other at the anterior margin of the acetabulum, and a strand of cells extending between the larger groups. The posterior group is the rudiment of the gonads and the anterior one of the copulatory organs. Only the major portion of the excretory system was observed. In immature larvae the tissues are fragile and disintegrate before the tubules are visible and in older ones the excretory ducts are obscured by the masses of glandular cells. Certain observations indicate that the flame cells are arranged in groups of three, but the complete pattern was not worked out. The system forms as separate left

and right components which meet and fuse at the posterior end of the body and the anterior portion of the tail. The two excretory pores are located on the sides of the tail, as shown in Figure 19. The vesicle is spherical to oval, thin-walled, and from either side a collecting duct passes forward, median to the digestive cecum, below the cecum at the level of the acetabulum, and then forward to the level of the oral sucker, where it recurves and passes posteriad. Near the middle of the body the recurrent duct divides into anterior and posterior branches. In the preacetabular area the collecting ducts have median and lateral branches and these branches subdivide in turn to form a ramified pattern as shown in Figure 20. In mature cercariae the collecting ducts contain spherical to oval concretions; in the postacetabular area the granules are larger, 0.005 to 0.01 mm. in diameter and more numerous, four or five at any level of the duct, whereas in the preacetabular ducts and their branches the granules are smaller, 0.003 to 0.005 mm. in diameter, and are arranged in single rows.

Himasthla mcintoshi n. sp.

This name is proposed for the seven specimens from the long-billed curlew, *Numenius americanus americanus*, collected by J. Bushman in Tooele County, Utah, on April 21, 1954 and deposited in the Helminthological Collection of the U. S. National Museum under the number 54,721. The species is named in honor of Dr. Allen McIntosh, Parasitologist in the Agricultural Research Service of the U. S. Department of Agriculture, in recognition of his contributions to helminthology and of the generous aid he has provided for other workers.

The worms are all sexually mature, with eggs in the uteri, chiefly in the initial one-half of the organ, with the terminal portion almost if not quite empty. Five of the worms are much bent or coiled and the one shown in Figure 16 is the only straight-bodied specimen. They vary from 6 to 11 mm. in length and from 0.5 to 0.7 mm. in greatest width. The anterior end bears a reniform collar which, in the two specimens in which they could be counted, is armed with 35 spines. There is a single row, interrupted ventrally, with 29 large spines, each 0.078 to 0.084 mm. in length and 0.02 mm. wide at the base, and at either end of the row, on the ventral side, there are three smaller corner-spines, about 0.055 mm. in length and 0.016 mm. wide at their bases. The lateral edges of the preacetabular region are curved ventrad and mediad, creating a median ventral depression (Fig. 17). When the body is contracted, the lateral walls are crenated and each annulus in the postacetabular region bears a circlet of small cuticular spines. In the preacetabular region the spines are closer together and arranged in an imbricated pattern, a cuticular arrangement characteristic of the genus *Himasthla*. The acetabulum is slightly less than twice its diameter from the anterior end of the body; it is directed antero-ventrad, protrudes slightly, and measures from 0.33 to 0.39 mm. in length and 0.31 to 0.35 mm. in width.

The mouth is subterminal, the oral sucker is 0.13 to 0.16 mm. in diameter, followed almost immediately by the pharynx, about 0.14 mm. long and 0.10 mm. wide. The esophagus extends almost to the acetabulum and the digestive ceca terminate blindly near the posterior end of the body.

The excretory pore is terminal, the vesicle is short and divides behind the posterior testis, with the collecting ducts passing forward just median to the

digestive ceca. They are clearly visible in the region between the anterior ends of the vitellaria and the acetabulum. Anterior to the acetabulum they are lateral in position and extend forward to the level of the oral sucker where they turn backward. Further details of the excretory system could not be observed.

The testes are oval, elongate, the posterior testis about its length from the posterior end of the body. There may be a short interval between the testes or they may be almost contiguous. The posterior testis is slightly larger than the anterior one and measures from 0.56 to 0.65 mm. in length and 0.22 to 0.28 mm. in width. Sperm ducts are not visible in the whole mounts; in most of the specimens the cirrus-sac extends about the diameter of the acetabulum behind that sucker; in the one shown in Figure 16, the cirrus-sac is coiled and consequently does not extend as far posteriad. The posterior one-half to two-thirds of the cirrus-sac is filled with a seminal vesicle and the anterior portion contains the eversible male duct, surrounded by secretory cells. The cirrus bears small recurved spines and the genital pore is median at the anterior margin of the acetabulum.

The ovary is spherical, 0.18 to 0.20 mm. in diameter, situated a short distance in front of the anterior testis. The oviduct arises at the posterior margin and the ootype and Mehlis' gland are posterior to the ovary. The vitellaria are lateral to and somewhat overlap the digestive ceca dorsally and ventrally. The follicles are spherical to oval, 0.04 to 0.065 mm. in diameter, continuous on both sides of the body, although somewhat reduced in one specimen at the level of the posterior testis. They extend from the posterior end of the body about two-thirds of the distance to the anterior end, terminating about two-thirds of the distance from the ovary to the acetabulum. Transverse ducts at the level of the ootype pass mediad to form a vitelline receptacle which discharges into the oviduct immediately before the ootype. The initial portion of the uterus is filled with spermatozoa. The eggs are broadly oval, those near the ovary average 0.100 by 0.076 mm., those farther along in the uterus are rounded, often collapsed, and may be slightly longer.

Himasthla mcintoshi agrees most closely with *H. rhigedana*, type of genus. Both are from species of *Numenius* and they are the only described species with more than 31 spines on the collar. Dietz reported a total of 34 to 38 spines in *H. rhigedana*, with 2, 3, or 4 corner-spines at each end of the row. In both of these species the corner-spines are very close together and often superimposed on those of the lineal row. Contractions of muscles in these locations produce variable orientation of the spines, so determination of their number and disposition is difficult. In Figure T of Dietz, the upper corner-spine on the left side could be interpreted either as a corner-spine or as the terminal spine in the collar-row. The two species differ in geographical distribution; *H. rhigedana* is from Arabia and *H. mcintoshi* from northwestern United States. Although the suckers do not differ greatly in size, *H. rhigedana* is more than twice as large as *H. mcintoshi*, the reproductive organs are much larger, although the eggs are smaller. The most obvious difference is in the disposition of the vitellaria; in *H. rhigedana* the vitellaria are interrupted at the testicular levels whereas in *H. mcintoshi* the follicles are continuous.

The type specimen is deposited in the U. S. National Museum Helminthological Collection under number 54,721.

Himasthla piscicola n. sp.

This species is based on specimens found by Dr. H. O. Ewert, veterinarian of the Zoological Society, Toledo, Ohio, in the alimentary canal of a fish, *Arapaima gigas,* from the Amazon River, Brazil. They were sent for determination to Dr. Leonard Allison of the Institute for Fisheries Research at the State Fish Hatchery, Grayling, Michigan. With the specimens there was the following information: "HISTORY; the 24 inch specimen arrived here in September. The fish ate in the first weeks, four to five goldfishes three inches long, daily, and came gradually down to one fish a day until he stopped eating around the 18th of December. In this week he vomited several small balls of mucus. Under the microscope, these balls appeared to consist of cells, mucus and many flagellates, *Octomitus intestinalis.* AUTOPSY; the abdominal investigation showed inflammation of the intestinal tract as well as the abdominal lining (peritonitis). The stomach lining was highly inflamed and congested. The stomach cavity was filled with a tenacious mucus and a certain parasite, which will be found separated on the accompanying slide." According to Allison (*in litt.*), the parasite was a trematode which had been mounted in water under a cover-glass and arrived perfectly dry. Other specimens were removed from the contents of the stomach which was preserved in water. Allison identified the worms as members of the genus *Himasthla.* Professor S. Yamaguti examined certain of the specimens and agreed with the generic determination, noting differences between these specimens and *H. tensa* Linton, 1940. Subsequently, Allison wrote Dr. G. R. La Rue, at the Animal Parasite Research Laboratory, Beltsville, Maryland, and sent him two of the worms in the belief that La Rue would write the description. But Dr. La Rue suggested that the writer examine the specimens and make the report. They are here described as a new species, *Himasthla piscicola.*

The two specimens measure 8.2 and 9.8 mm., respectively, in length. The larger one, shown in Figure 15, is designated as type. The organs of the smaller worm are almost as large as those of the type specimen. In the smaller one there are masses of spermatozoa in the initial portion of the uterus but no eggs. There are two eggs, one of them collapsed, in the uterus of the larger worm. In these specimens the reniform collar, open ventrally, delimits a short, flattened area at the anterior end of the body. The collar-spines are intact but other spines have been lost. There are 29 spines on the collar, 25 in the lineal row and two on either side behind the terminal ones. Those at the ventral corners are as large as those in the lineal row; they measure 0.085 mm. in length and 0.025 in maximum width. Behind the collar there is a short, neck-like constriction. The specimens are much extended, a result of their protracted immersion in water, and the uterine region between the cirrus-sac and the ovary is especially narrow. In the larger worm the width at the collar is 0.875 mm. The acetabulum is 0.44 mm. long and 0.50 mm. wide; it is about its diameter behind the collar. The oral sucker protrudes slightly and measures 0.18 by 0.19 mm. The pharynx is large, 0.24 mm. long and 0.11 mm. wide. The esophagus extends to the level of the acetabulum and the digestive ceca end blindly near the posterior end of the body. The testes are situated much nearer the middle than the posterior end of the body.

They partially overlap, the posterior third of the anterior testis is in the same zone as the anterior third of the posterior testis. They are elongate, slightly notched; the anterior one is 0.875 mm. long and 0.20 mm. wide, the posterior one is 0.89 mm. long and 0.20 mm. wide. The cirrus-sac extends posterior to the acetabulum more than twice the diameter of that sucker. A large seminal vesicle occupies the posterior half of the sac; the anterior portion of the vesicle and the succeeding duct are enclosed in a large, many-celled prostate gland. The cirrus is not protruded and no spines were observed. The ovary is situated near the middle of the body, only a short distance in front of the anterior testis, but would be relatively more posteriad if the uterus were filled with eggs. It measures 0.18 by 0.20 mm. The ootype complex is large, situated immediately posterior to the ovary; the initial portion of the uterus is filled with spermatozoa and there are two eggs in the uterus. One is collapsed, the other measures 0.114 by 0.064 mm. The vitelline follicles are continuous on each side of the body and extend from the posterior ends of the digestive ceca about three-fourths of the distance from the ovary to the posterior end of the cirrus-sac. The follicles would probably extend farther forward in more mature individuals.

Although the specimens are not mature, *H. piscicola* differs from all other adequately described species with 29 collar-spines in the position of the gonads, the shape and overlapping arrangement of the testes, and in the relative length of the posttesticular region of the body. *Himasthla piscicola* and *H. annulata* were found in the digestive tract of fishes from the Amazon River; it is possible that the two are identical, that some avian species is the natural host, and that the discovery of these worms in fish hosts is entirely incidental. The worms are similar in size, but the description and figures of Diesing give no information concerning internal morphology of *H. annulata* and it is quite impossible to determine whether the two are identical.

The type specimen of *H. piscicola* is deposited in the Helminthological Collection of the U. S. National Museum under the number 39,445.

Discussion

An investigation conducted by the U. S. Bureau of Commercial Fisheries is attempting to determine the causes for the decline in populations of *Mya arenaria* along the coast of New England and possible biological measures for control of the principal predators, the green crab (*Carcinus maenas*) and the horseshoe crab (*Limulus polyphemus*). *Mya arenaria* harbors the sporocysts and cercariae of *Cercaria myae* Uzmann, 1952; the larval stages of an as yet undetermined species of *Gymnophallus* (Stunkard and Uzmann, 1958). The palps and gills contain metacercariae of digenetic trematodes. Since the asexual generations of these metacercariae must occur in mollusks which live in the immediate vicinity of the infected clams, a survey of the more abundant species and those most likely to carry the trematode infections, is in progress. Furthermore, since the definitive hosts of these metacercariae are animals that feed on *M. arenaria*, examination of shore-birds has been started. To obtain precise information under controlled conditions, metacercariae from *M. arenaria* have been fed to laboratory-reared eider ducks,

herring gulls, common terns, white mice, and golden hamsters. The results, together with other pertinent information, are presented in this paper.

The metacercariae from *M. arenaria* proved to be larvae of three different species, all in the genus *Himasthla*, and the adults recovered from *L. argentatus* have been of value in resolving taxonomic problems in the genus. Typically, echinostomes are parasites of warm-blooded vertebrates and members of *Himasthla* have metacercarial stages in marine mollusks and mature in avian hosts. *Himasthla ambigua* Palombi, 1934 was described from metacercariae found in *Tapes decussatus* from the Gulf of Naples, but the adult stage is yet unknown. Palombi reported that the worms have 32 cephalic spines, that the infection is seasonal, and suggested that a bird, perhaps a migrant, is the final host. Elucidation of the life-cycle of *H. compacta* supports previous belief that species of *Himasthla* are parasites of birds and that the larvae occur in marine mollusks. Although adults of *H. compacta* are less than one-half the size of those of *H. quissetensis*, the cercariae of the two species are almost identical in size.

Since members of *Himasthla* typically are parasites of shore-birds, it is surprising that four species have been recorded from abnormal hosts, three from fishes and the fourth from a fruit-eating pigeon. Two species, *H. multilecithosa* from the pigeon and *H. piscicola* from *Arapaima gigas*, were taken from captive hosts and the time and place of infection are unknown. The finding of specimens of *Himasthla* in the digestive tract of marine and fresh-water fishes presents a biological anomaly. The fishes could have ingested a bird or its entrails that had fallen in the water and the proposed identity of *H. tensa* and *H. elongata* suggests such an explanation. *H. annulata* and *H. piscicola* were taken from the alimentary tract of fishes from the Amazon River of Brazil, and if the worms found in *A. gigas* were acquired in South America, they must have persisted for an unusually long time in the fish. The specimens were still juvenile, which indicates that they were recently ingested or that they fail to attain sexual maturity in the cold-blooded host. Since *H. annulata* (Diesing, 1850) and *H. piscicola* are from Amazonian fishes, it is possible that the two are identical, that some avian species is the natural host, and that the discovery of these worms in the digestive tract of fishes is entirely incidental.

Summary

The validity of species in the genus *Himasthla* is discussed; *Echinostoma annulatum* (Diesing, 1850) is transferred to *Himasthla* and *H. tensa* Linton, 1940 is suppressed as a synonym of *H. elongata* (Mehlis, 1831). A specimen from *Nycticorax nycticorax*, tentatively assigned to *H. elongata*, is very similar to *H. secunda* (Nicoll, 1906), which suggests the possibility that *H. secunda* may be a not-fully mature form of *H. elongata*. Three new species are described; *H. mcintoshi* from *Numenius americanus americanus* taken in Tooele County, Utah; *H. piscicola*, probably an accidental infection, from the South American fish, *Arapaima gigas;* and *H. compacta* from experimental infection of the herring gull, *Larus argentatus*. The life cycle of *H. compacta* has been traced; the asexual generations occur in *Hydrobia minuta*, the cercariae encyst in *Mya arenaria* and probably other mollusks.

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THE SEASONAL OCCURRENCE OF MYTILUS EDULIS ON THE CAROLINA COAST AS A RESULT OF TRANSPORT AROUND CAPE HATTERAS[1]

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The edible mussel, *Mytilus edulis* L., attaches to rocks, pilings, and other firm substrates, being particularly abundant in the lower intertidal zone, where it is a dominant organism in the community (Newcombe, 1935; Dexter, 1947). A temporate and boreal species, it is found on both American and European coasts of the North Atlantic as well as Asian and American coasts of the North Pacific Ocean. Its distribution has aroused the interest of ecologists as an example of the effects of temperature on determining the geographical limits of species. Hutchins (1947) has suggested that the southern limit of *M. edulis* occurs where the mean sea surface temperature during the warmest month is approximately 80° F. (26.6° C.).

In the western Atlantic, *M. edulis* reaches its southern limit in the Carolinas. Although Hutchins showed the 80° summer isotherm intersecting the coast in the vicinity of Cape Hatteras, the edible mussel has been reported as far south as Charleston, South Carolina, three hundred miles below Cape Hatteras. This apparent contradiction of Hutchins' temperature limitation of *M. edulis* is discussed and related to the biology of the species. Its occurrence on the North Carolina coast is analyzed in relation to coastal water masses, their temperature and movement, and meteorological conditions.

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Observations

Mytilus edulis has been collected in the course of study of littoral organisms in the Beaufort and Cape Hatteras areas (Fig. 1). In the latter region, this study has centered on fauna attached to a group of eight wrecks near the outer beach. All but one wreck ordinarily extend above the water surface; all are metal and provide a firm substrate for many sessile organisms. *Mytilus edulis* occurs all year long on wrecks north of Cape Hatteras (stations 1–6: Kitty Hawk, Overlook, Pea Island, Rodanthe, Waves, and Salvo), where it is the dominant organism in the lower part of the intertidal zone and extends into deeper water on subtidal surfaces.

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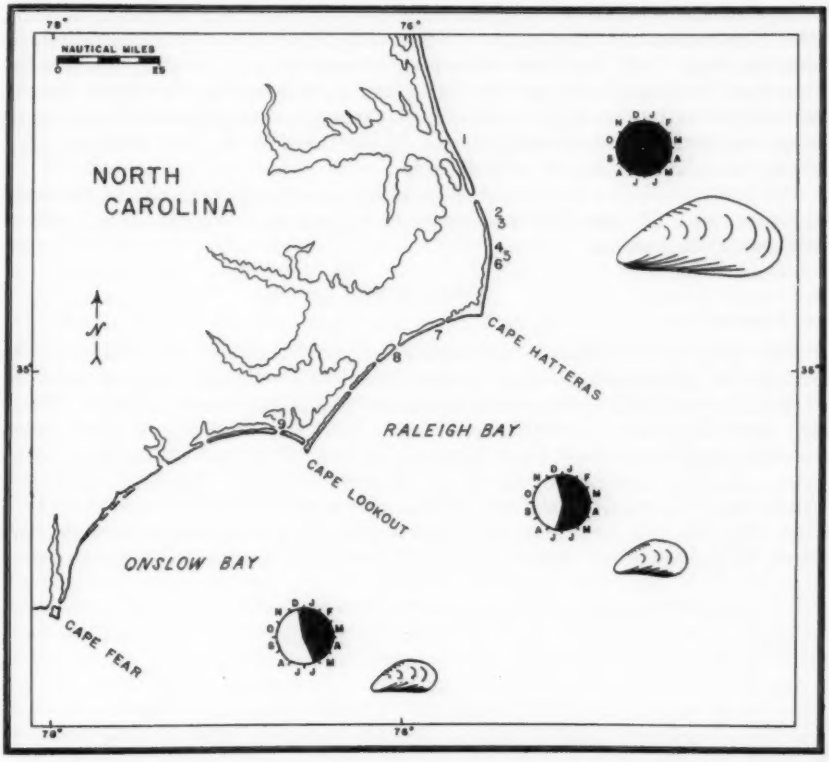

FIGURE 1. Chart of North Carolina coast, showing stations for the collection of *Mytilus edulis*, the portion of the year that *M. edulis* is present in each region, and the relative size attained by this species in each region. The blackened portion of the clock symbol indicates the presence of *M. edulis*. Station 1—Kitty Hawk, 2—Overlook, 3—Pea Island, 4—Rodanthe, 5—Waves, 6—Salvo, 7—Ocracoke Island, 8—Portsmouth Island, 9—Beaufort.

Here, it attains a maximum size of at least 75 mm. However, on the two wrecks in Raleigh Bay, south of Cape Hatteras (Ocracoke and Portsmouth, stations 7 and 8), *M. edulis* has been found only in spring, disappearing in late July. The maximum size attained in Raleigh Bay is 33 mm.

In the Beaufort area (station 9), *M. edulis* has been collected on rock jetties and pilings near Beaufort Inlet, where it shares its intertidal habitat with *Brachidontes exustus*, a small ribbed mussel which is distributed from North Carolina to the West Indies. Variable in abundance from year to year, *M. edulis* in the Beaufort area is often relatively scarce, with a few individuals scattered among large populations of *B. exustus*. Occasionally, however, it is very abundant in the spring, far outnumbering *Brachidontes*. While adequate records over a long period of time have not been kept, it is known that the appearance of *M. edulis*

in large numbers occurs at irregular intervals. It was very abundant in June, 1938; Stephenson and Stephenson (1952) noted that it was common locally in April and May, 1947; and there have been several years in the past decade when it has been abundant in the spring. *M. edulis* appears during the winter months. Ordinarily it disappears in June, leaving *B. exustus* as the predominant mussel on pilings and jetties in the intertidal zone. In the Beaufort area, *M. edulis* is small, attaining a maximum size of only 30 mm.

While *M. edulis* is a permanent resident and a dominant organism of the intertidal zone north of Cape Hatteras, the niche it occupies is largely taken south of the Cape by *B. exustus*.

Discussion

Temperature

The differential success of *M. edulis* on the North Carolina coast can be attributed to differences in water temperatures. Two distinct types of water lie off the coast, exhibiting different temperature and salinity characteristics. These have been designated (Bumpus and Pierce, 1955) as Virginian Coastal water, extending northward from Cape Hatteras to Cape Cod, and Carolinian Coastal water, extending southward from Cape Hatteras to Cape Canaveral. Virginian Coastal water is much colder than that of the more southern Carolinian Coastal water (Fig. 2). At Cape Hatteras, there is typically a sharp temperature gradient (Parr, 1933), and these water types remain relatively distinct, with little mixing.

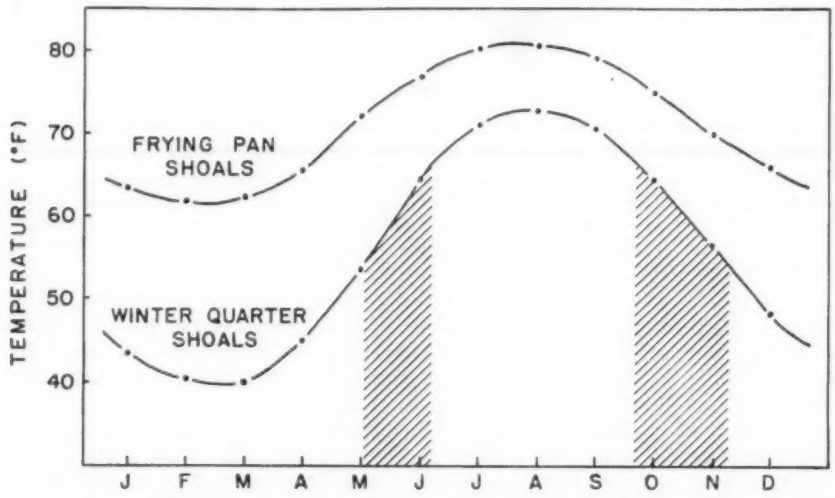

Figure 2. Monthly means of sea surface temperature at Frying Pan Shoals Lightship (Carolinian Coastal water) and Winter Quarter Shoals Lightship (Virginian Coastal water). Shading indicates periods of temperature suitable for attachment of *Mytilus edulis* in the southern part of Virginian subprovince. Data for 1947–1956 for Frying Pan Shoals and 1925–1940 for Winter Quarter Shoals from Bumpus (1957a).

Biologically, these temperature conditions are extremely important, determining which species survive along each part of the coastline. The temperature gradient in the Cape Hatteras area helps to separate the fauna of the Virginian biogeographic subprovince from that of the Carolinian subprovince. On the basis of their respective temperature regimes, the Virginian subprovince can be classified as a temperate zone (8–25° C., 46–77° F.), and the Carolinian subprovince can be classified as a sub-tropical zone (15 to almost 30° C., 59–85° F.) in the classification system of Vaughan (1940).

South of Cape Hatteras, summertime maximum temperatures may exceed the thermal limits of *Mytilus edulis*. Ritchie (1927) found that this species was killed by a fourteen-hour exposure to 84° F., and Bruce (1926) has indicated that it could not survive more than one hour at 86° F. We have recorded water temperatures as high as 84° F. along the shore of Raleigh Bay in August, 1959, and Maturo (1959) recorded a similar temperature in the Beaufort area in August, 1954. These are not temperatures of estuarine water that had warmed over shallow inshore areas; they represent oceanic conditions to which mussel populations would be exposed. These summertime temperatures are sufficiently high to cause the death of any edible mussels south of Cape Hatteras. Death in nature may precede the attainment of the high temperatures cited.

The annual date of disappearance from the natural habitat is advanced or delayed in relation to the progression of the temperature cycle. For example, in the spring of 1956 when mean air temperatures in eastern North Carolina were several degrees lower than average (U. S. Weather Bureau, 1956), and water temperatures along the middle Atlantic Coast were similarly depressed (Bumpus, 1957b), the delay in vernal warming permitted *Mytilus edulis* and many other organisms of the winter sequence to persist in the Beaufort area well into July. In the Beaufort area, the disappearance of mussels more closely coincides with the attainment of a water temperature of 80° F. It seems likely that higher air temperatures and solar radiation raise the temperature of mussels to a critical level during their exposure at low tides. Under these conditions, the 80° isotherm chosen by Hutchins to represent the southern limit of *M. edulis* serves better than 84° as an empirical expression of the ecological requirements of this species. The different dates of disappearance recorded for the Beaufort area (mid-June) and for the Ocracoke area (late July) reflect a later date for the attainment of lethal high temperatures in the more northern Ocracoke area.

North of Cape Hatteras, such high temperatures do not occur in the ocean, and *M. edulis* survives the maximum temperatures of summer. Just as an upwelling of cool bottom water permits *M. edulis* to live on the coast of Lower California, Mexico (Woods Hole Oceanographic Institution, 1952), an upwelling of cool subsurface water contributes to the maintenance of a suitable environment for this species north of Cape Hatteras (Wells and Gray, 1960). The occurrence of this cooler water north of the Cape permits the maintenance of sizable populations of other northern species with boreal affinities (Wells *et al.*, 1960).

Water movements

In view of the fact that Carolinian Coastal water is too warm to permit year-round survival south of Cape Hatteras, *Mytilus edulis* in this region must owe its

existence to larvae originating from the extensive populations north of the Cape, which could renew colonies south of the Cape, if they were provided suitable transport. However, while it is recognized that a southward-flowing coastal current follows the edge of the continental shelf off the middle Atlantic Coast, this coastal current normally does not pass Cape Hatteras; instead, it turns seaward and parallels the Gulf Stream, flowing in a northeasterly direction (Bigelow, 1933; Ford *et al.*, 1952). The Gulf Stream System approaches Cape Hatteras from the south and then flows northeastward, away from the North American mainland (Iselin, 1936). Because the Outer Banks of North Carolina project across much of the continental shelf, the average position of the inner edge of the Gulf Stream lies only ten miles off the point of Cape Hatteras (Marshall, 1951), and the Gulf Stream System has a marked effect on this part of the coastline. As a result of the convergence of these two currents on the Cape Hatteras region, there is a pronounced offshore movement of water. Bumpus (1955) noted the offshore movement of several drift bottles released in 1950 in the Cape region. Such conditions would appear to bar the movement of mussel larvae around Cape Hatteras; instead, the larvae would be carried out to sea.

Although there is no regular movement of water around the Cape, Bumpus and Pierce (1955) witnessed the breaching of this barrier and postulated the sporadic occurrence of transient indrafts of Virginian Coastal water into Raleigh Bay, as the result of northeast storms in the Cape Hatteras region. The duration of a northeast storm and the subsequent weather pattern apparently would determine the fate of this parcel of Virginian Coastal water. If the storm lasts but one or two days, this water may eventually be absorbed within the Carolinian Coastal water by mixing, or a violent meander of the Gulf Stream may sweep over the continental shelf, pushing or drawing the Virginian Coastal water to the northeast. If the northeast storm persists or is followed closely by another northeast storm, the parcel of Virginian Coastal water may grow in size and be driven farther southward, around Cape Lookout into Onslow Bay. Northeast storms, common in fall and winter months, are a characteristic component of the climate on this coast. Strong northeasterly winds push water southward past the point of Cape Hatteras. By the mechanism described by Chase (1959) concerning the effect of wind on water movement, wind tides produced north of the Cape help to move Virginian Coastal water across Diamond Shoals.

Bumpus and Pierce (1955) found Virginian Coastal water in Raleigh Bay in January, 1954, after a three-day storm. A similar parcel of water with the characteristics of Virginian Coastal water was noted at Frying Pan Shoals Lightship in November, 1956, following an extended period of northeast storms in the Hatteras area (Bumpus, 1957b). Again, for April, 1958, Day (1960) recorded the occurrence of Virginian Coastal water at Frying Pan Shoals, 145 miles southwest of Cape Hatteras. Bumpus (1957b) noted that this southward movement of water had progressed at the rate of seven or eight miles per day. At the height of a storm, water has been observed flowing past the point of Cape Hatteras at a rate of one to two knots. After having paralleled the coastline north of the Cape, it continues to flow in a southward direction rather than following the coast westward.

Bumpus and Pierce suggested that this southward movement of water might distribute northern planktonic elements southward along the coast, and that this might account for the presence of anomalous species of northern affinities in the winter-spring sequence in Onslow Bay. Both Williams (1948, 1949) and Sutcliffe (1950) had found a number of northern species in this area whose occurrence was apparently restricted to the cold months of the year. *Mytilus edulis* appears to be such an anomalous species in the Carolinian subprovince. Larvae of this species from north of Cape Hatteras could be distributed along the coast south of the Cape by such a water movement.

Settlement period

To be effective in the transport of *Mytilus edulis* larvae, these storms must occur during the reproductive period of the mussel. The period of breeding and attachment of this species occurs at different times in different parts of its range, apparently in response to temperature conditions. Engle and Loosanoff (1944)

TABLE I

Attachment of Mytilus edulis *at various locations*

Locality	Beginning		Maximum		Reference
	Date	Temp. (° C.)	Date	Temp. (° C.)	
Lamoine, Me.	June (late)	13	July (end)	15	Fuller, 1946
Milford, Conn.	June 1	12	June 15	15	Engle and Loosanoff, 1944
Oakland, Calif.	March	14	May	18	Graham and Gay, 1945
Kola Fjord, USSR	July	12	—	—	Zenkevitch, 1935
Millport and Caernarvon, U.K.	July	12	—	—	Bengough and Shepheard, 1943
Plymouth, U.K.	June	13	—	—	Mott, 1944
Kanazawa, Japan	January	10	May	17	Miyazaki, 1938

found *M. edulis* settling in early June at Milford, Connecticut, when the water temperature was 12.5° C. (about 54° F.). Attachment reached a peak about a month later, then fell off when water temperatures reached 19° C. (66° F.). After a brief halt in the first half of August, some attachment occurred in late August, when water temperatures were declining from the seasonal maximum. A comparison of the dates of attachment from other studies (Table I) shows that attachment typically follows the pattern observed at Milford, beginning near 12° C. (54° F.) and reaching a maximum at 15 to 18° C. (59–64° F.).

In the southern part of the Virginian subprovince, there appear to be two important periods of settlement of *Mytilus* larvae, one in late spring and one in late fall, well separated by a warm period when little or no settling occurs. Spring attachment may begin in April, when water temperatures warm to about 54° F., and extend into early June. Optimum conditions are again realized in October and November, when water temperatures are between 66 and 54° F. These temperature intervals are interposed on the graph of water temperatures at Winter

Quarter Lightship in Figure 2. Evidently, the late fall attachment period is more important than the late spring attachment period for colonizing the northern part of the Carolinian subprovince. Northeast storms are more likely in the October-November period than in late spring, and mussels which attach in May are soon killed by high temperatures south of the Cape. Only the late fall set would have the opportunity to grow to sizable dimensions. *Mytilus edulis* has been noted in the Beaufort area starting in December with representatives of the fall set. Only representatives of this set could reach a length of 30 mm. by the time the summer mortality sets in. Therefore, northeast storms in fall months should be primarily responsible for the appearance of mussel populations south of Cape Hatteras.

Correlation with weather records

From the above discussion, it would appear that the occurrence of *Mytilus edulis* south of Cape Hatteras can be directly related to movements of water around the Cape resulting from northeast storms. However, to be effective these storms must occur during the fall reproductive period for this species. An examination of the cycle of monthly mean water temperatures shows that this period ordinarily occurs in October and November (Fig. 2), but warmer than average conditions in September will extend the attachment period into December, and cooler than average conditions in September will make the latter part of that month suitable

TABLE II

Northeast storms in the Cape Hatteras area suitable for the transport of Mytilus edulis *larvae south of Cape Hatteras, 1954 to 1958, with relative abundance of* Mytilus edulis *at Beaufort the following spring*

	Number of days	Dates	Mean wind velocity (mph)	*Mytilus edulis*
1954	—	none	—	none
1955	3	October 2–4	14.0	rare
1956	7	October 10–16	17.7*	
	3	October 19–21	14.5	
	6	October 24–29	20.4*	abundant
	5	November 2–6	15.3	
	—			
	21			
1957	3	October 3–5	10.8	moderately
	5	October 11–15	13.0	abundant
	—			
	8			
1958	4	October 6–9	16.9	
	4	October 18–21	10.6*	abundant
	—			

* Severe storm; tides 2 to 3 feet above normal.

for attachment. Bumpus and Pierce noted that a short-lived storm is not effective in causing a significant penetration of water south of Cape Hatteras, and suggested that a storm that lasted for three days or more was necessary to produce the southward movement. With these criteria in mind, weather records for Cape Hatteras (Local Climatological Data) have been examined for the occurrence of northeast storms during the five-year period from 1954 to 1958. Only those storms were counted in which a northeasterly wind (NNE, NE, or ENE) predominated three or more days, with a mean velocity in excess of 10 mph. Using water temperature records by Bumpus (1957a, 1957b) and Day (1958, 1960) as a guide to the late fall attachment period of *M. edulis,* the northeast storms which could have carried mussel larvae south of Cape Hatteras during this five-year period are listed (Table II).

Mean wind velocity is included in Table II as a measure of the likely effectiveness of each storm in moving Virginian Coastal water southward. While 1958 storms extended over as many days as 1957 storms, a greater severity and effectiveness of the 1958 storms is indicated by a greater wind velocity. The fall of 1956 was distinguished by a remarkable series of storms in October and early November, with only brief intermissions. It was after these storms in 1956 that a parcel of Virginian Coastal water was detected off Frying Pan Shoals.

Relative abundance of *Mytilus edulis* at Beaufort the following June is also included in Table II. Although *M. edulis* may comprise up to 80% of the mussels on the jetties and pilings, such populations are uncommon. Ordinarily, it comprises less than 10% of the mussels, the great majority being *Brachidontes exustus.* If *Mytilus edulis* colonies in the Beaufort area owe their existence to a transport of larvae around Cape Hatteras by northeast storms the preceding fall, a close correlation would be expected between the number and severity of northeast storms and the abundance of *M. edulis* at Beaufort the following June. A comparison of these items in Table II shows a very close correlation. Not only does the occurrence of *M. edulis* agree quantitatively with the prior occurrence of northeast storms, but this species was not found in the spring following an exceptionally storm-free fall season in 1954. The greatest abundance of mussels was recorded after the remarkably stormy fall of 1956. The abundance of mussels also reflected the relative severity of storms in the fall of 1957 and 1958, mussels being more common after the 1958 season. It seems evident that this is a cause-effect relationship.

The presence of *Mytilus edulis* in the northern part of the Carolinian subprovince serves as an indication of the southward penetration of Virginian Coastal water. Consequently, the presence of *M. edulis* at Charleston, South Carolina, indicates that Virginian Coastal water may be driven this far south.

Other species

Other molluscs of northern affinities probably owe their sporadic appearance in the Beaufort area to a similar transport of their larvae southward around Cape Hatteras by northeast storms. For example, although the normal range of *Littorina saxatilis* in the western Atlantic extends from the Arctic to Cape May, New Jersey (Abbott, 1954), Stephenson and Stephenson (1952) reported that species in the Beaufort area during April and May, 1947. The anomalous appear-

ance in the Beaufort area suggests that it, too, was transported southward around Cape Hatteras in larval stages. At Cape Ann, Massachusetts, Dexter (1947) noted spectacular fluctuations in abundance of *L. saxatilis* for year to year in the same location. Such variation in numbers indicates that larvae of this species are subject to water movements, and that they may be carried some distance from the parent colony. Its appearance in Beaufort would be in keeping with this type of distribution.

Summary

Although *Mytilus edulis* survives year-round north of Cape Hatteras, summer water temperatures are high enough to kill it south of that point. Populations of this species in the Carolinian subprovince are apparently maintained by an external source of larvae. Larvae can be transported around Cape Hatteras by southward movements of Virginian Coastal water that result from persistent strong northeast winds in late fall. The presence and abundance of *M. edulis* at Beaufort has been correlated with the abundance and severity of northeast storms occurring in the Cape Hatteras region during the preceding fall, over a five-year period. Probably other northern species owe their sporadic appearance in the Beaufort area to similar transport of larvae by southward water movements.

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THE INFLUENCE OF TEMPERATURE ON OSMOTIC REGULATION IN TWO SPECIES OF ESTUARINE SHRIMPS (PENAEUS)

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Estuarine decapod Crustacea living in an environment of varying salinities and temperatures face great osmoregulatory problems. Presumably such animals could either possess well-developed osmoregulatory powers or, lacking these, follow passively the fluctuations of the external medium. Migratory crustaceans present a special case, for their movements may carry them in and out of estuaries, often quite rapidly, thus complicating their osmoregulatory work.

Among swimming shrimps (Penaeidae) are many which live a wandering life, and on the southeastern and Gulf coasts of the United States three such species, *Penaeus setiferus* (Linn.), *P. duorarum* Burkenroad and *P. aztecus* Ives, occur in enormous numbers in and near estuaries.

The geographic ranges, life histories, migratory habits and general ecology of these species have been reported in whole or in part in numerous publications, including: Burkenroad (1934, 1939); Gunter (1950); Heegaard (1953); Lindner and Anderson (1956); Pearson (1939) and Williams (1955). It is well established that these species spawn at sea. The demersal eggs hatch into nauplii which undergo larval transformations while being transported shoreward; by the time they are transformed to the postlarval stage, they are entering estuaries where they proceed to shallow brackish areas to continue growth. With increasing age they move into deeper estuarine waters, return to sea with approaching sexual maturity, and seldom, if ever, return to the estuaries. The estuarine phase lasts about four months in summer but is extended in colder months.

Knowing this, the questions asked in this study are: do these animals regulate their internal salinities and, if so, how well do they accomplish osmoregulation in a variety of salinity and temperature combinations?

Similar questions have been posed by others. Panikkar (1941) found that *Leander serratus, L. squilla* and *Palaemonetes varians* in undiluted sea water are able to maintain their internal fluids at lower concentrations than the outside medium (hypotonic regulation), and in waters of low-salt content are able to maintain their internal fluids at higher concentrations than the outside medium (hypertonic regulation). Panikkar and Viswanathan (1948) found the same ability exhibited by *Metapenaeus monoceros* and Panikkar (1951) reported it shown to a lesser extent in *M. dobsoni, Penaeus indicus* and *P. carinatus* and in the grapsoid crabs *Eriocheir sinensis* and *Varuna litterata*. Panikkar (1951: page 171) states that this ability to regulate is "probably the most elaborate osmotic mechanism ever perfected by invertebrates" and is widely distributed among the

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Crustacea. Broekema (1941) and Verwey (1957) have shown that the blood of *Crangon crangon* is hypotonic in sea water and hypertonic in diluted sea water. Similar osmoregulatory patterns in a number of grapsoid and ocypodid crabs have been found by Edmonds (1935), Jones (1941), Gross (1955, 1957a) and in *Birgus latro* by Gross (1955).

The relationship of osmoregulation to temperature has not been so extensively investigated. Broekema (1941) found in *Crangon crangon* that young in summer tolerate lower salinities than adults. Adults seek higher salinities in cold weather and lower salinities in warm weather. The young, after a period of estuarine existence, go to sea in the fall and return to estuaries as adults the following summer, but they never again inhabit waters of as low salinity as they experienced when young. Broekema found that continued dilution of the external medium brought about continued dilution of the internal medium within limits, but in a constant salinity the concentration of blood increased with a fall in temperature.

Materials and Methods

Though three species of *Penaeus* are abundant in North Carolina, only two, *duorarum* and *aztecus,* are available in large numbers in nursery areas near Morehead City each year. These two species were used in this study. From spring to late summer *aztecus* is abundant on the nursery grounds and from midsummer to fall and winter *duorarum* is available. Because a wide range of sizes is represented in both species during the year and because shrimp of different sizes occupy different habitats, each species was divided arbitrarily into two groups, juveniles (under 100 mm. total length) and subadults (100 mm. and over). Only *aztecus* attained the larger sizes during the experimental period.

The problem of osmoregulation in these penaeids was approached as simply as possible. Freezing point depression (melting point) of blood was used as an indication of the ability of these animals to osmoregulate in various dilutions of sea water at different temperatures. Using modifications of the methods of Jones (1941), Gross (1954) and Hickman (1959) for determining melting points, small, uniform samples (approximately 1 $mm.^3$) of blood were taken in dried, thin-walled capillary tubes (1 mm. diameter), the ends of the tubes sealed with Vaseline, and the samples quick-frozen on dry ice. Samples were stored in a freezer until the melting points could be determined. Melting points of known standards of pure and salt water were determined with the aid of a differential thermometer.

Later, determinations of the unknowns were made by timing the end points of melting of the frozen crystals in the unknowns and standards as they warmed slowly in a waterproof, insulated box filled with ice brine, and by converting the melting times to degrees C.

Samples of blood were taken from individual shrimp by puncturing the sternum of the first abdominal segment with a pin or sharp awl, then catching the fluid in a capillary tube. Prior to making the puncture, the sternum and adjacent structures were thoroughly dried with absorbent cotton swabs. In large individuals, 70 mm. and above, fluid welled out and entered easily into the tubes by capillarity. In younger individuals the amount of fluid was often so small that it had to be sucked into drawn capillary tubes with the aid of a vaccine bulb. Most shrimp

stood this treatment well (though a few small individuals were lost) and the wounds closed readily.

Experimentation was limited to juvenile and subadult shrimp, and experimental conditions for salinity and temperature approximated those found in the sounds seasonally, where salinity ranges from about 7‰ to 34‰ and temperature from 4° to 30° C.

Five series of round, wide-mouthed, five-gallon jars were used as aquaria. Each series consisted of five jars containing salinities of approximately 10, 15, 20, 25 and 30‰ sea water (hydrometer readings). Dilutions were made with tap water. Water was aerated for 24 hours at room temperature before animals were introduced. At the beginning of an experiment shrimp were transferred from outside holding tanks supplied by a running sea water system to the jars, four individuals per jar. (The system pumps 30–34‰ Sound water through hard rubber and polyethylene pipes.) Shrimp in almost all cases were caught a day or two before each experiment began. Prior to each experiment samples of water and blood from shrimp were taken from the outside holding tanks. One blood sample was taken at random from shrimp in each jar after shrimp were placed in jars for two to three hours at room temperature (25–32° C.). Shrimp sampled were returned to the jars. (The few specimens badly injured or killed in the initial sampling were discarded and replaced.) At this same time a water sample in a capillary tube was taken from each jar as a check on the salinity mixtures. After samples were taken, four series were subjected to gradually lowered temperatures in a cold room. The fifth series was maintained as a control at room temperature throughout the experimental period. In 48 hours the temperature was lowered from about 28° to 18° C. and another round of blood samples was taken; in 96 hours at 8° C. another round was taken. At the end of 96 hours all animals were discarded, the water thrown out, new mixtures prepared, and fresh shrimp used for replications. Animals were not fed during the experimental periods.

A number of difficulties were experienced with the technique described above. It is desirable to have the brine solution warm slowly, one degree C. per half hour (Gross, personal communication). I had trouble maintaining this rate of warming, even when the box was precooled, for on hot humid days (temperature near 32° C. and humidity near saturation) the box would sometimes warm at a rate of one degree in fifteen minutes. Securing samples of uniform volume in the initial round of samples each week in this climate in summer was difficult. At temperatures above 22° C. shrimp blood coagulates rapidly, almost on contact with glass. The initial blood samples were taken from animals in air temperatures of 27°–32° C. and extreme difficulty was experienced in securing unclotted and uniform samples. As a corrective measure initial sampling was done in the cold room which had been quickly cooled to air temperature of 18° to 22° C. (water in the aquaria remained at approximately 28° C.). This method helped but still the amount of clotting hindered taking samples of uniform volume. This lack of uniformity in sample size gave somewhat distorted results in terms of melting points, but replications smoothed the inequalities. The 48- and 96-hour samples were readily taken at the lower temperatures.

For the experimental series, replications of individual determinations for both blood and water were averaged (Table I). The average values were subjected to

standard methods for linear regression analysis (Snedecor, 1956) in which melting point of blood was plotted against melting point of water (Figs. 2, 3 and 4). Each of the curves if extended to lower and higher salinities would probably assume a more or less sigmoid shape (Jones, 1941; Verwey, 1957), but in other crustaceans previously studied a considerable portion of the curve in the salinity range treated here, 10‰–30‰, is essentially a straight line. In this investigation a linear relationship for salt concentration of blood in this range of salinities is assumed.

Regression coefficients were subjected to analyses of variance and all except one were found significant at the 5% level. The one exception (*duorarum* experimental and control at 28.4° C.) which falls slightly short of this level of significance is attributed to sampling difficulty. If all regression coefficients are considered significant, an important question remains as to whether or not the lines in each figure

TABLE I

The number of determinations and their average values for the blood melting points shown in Figures 2, 3 and 4

		Mean salinity in ‰				
		10.06	15.11	20.17	25.09	30.50
P. aztecus 42–100 mm.						
Exp. & Contr. 28.3° C.	N Determinations	21	23	22	22	23
	$\bar{x}$ Melting point ° C.	−1.21	−1.33	−1.48	−1.56	−1.52
Exp. 48 Hrs. 16.2° C.	N	17	20	20	20	18
	$\bar{x}$	−1.10	−1.29	−1.34	−1.46	−1.57
Contr. 48 Hrs. 28.6° C.	N	4	3	4	4	4
	$\bar{x}$	−1.04	−1.26	−1.45	−1.56	−1.52
Exp. 96 Hrs. 8.8° C.	N	8	18	19	19	20
	$\bar{x}$	−1.13	−1.29	−1.41	−1.53	−1.75
Contr. 96 Hrs. 28.8° C.	N	5	6	6	5	6
	$\bar{x}$	−1.23	−1.33	−1.46	−1.45	−1.56
P. aztecus 120–150 mm.						
Exp. & Contr. 28.3° C.	N	10	10	9	9	8
	$\bar{x}$	−1.36	−1.44	−1.57	−1.69	−1.76
Exp. 48 Hrs. 16.2° C.	N	5	7	8	8	8
	$\bar{x}$	−1.22	−1.26	−1.41	−1.48	−1.58
Exp. 96 Hrs. 8.8° C.	N	2	4	7	7	8
	$\bar{x}$	−0.99	−1.24	−1.34	−1.56	−1.77
P. duorarum 35–100 mm.						
Exp. & Contr. 28.4° C.	N	24	26	26	26	26
	$\bar{x}$	−1.48	−1.45	−1.47	−1.54	−1.65
Exp. 48 Hrs. 17.8° C.	N	22	24	23	24	21
	$\bar{x}$	−1.32	−1.35	−1.47	−1.59	−1.65
Contr. 48 Hrs. 28.4° C.	N	6	6	5	6	6
	$\bar{x}$	−1.45	−1.51	−1.49	−1.62	−1.65
Exp. 96 Hrs. 8.75° C.	N	24	24	24	23	24
	$\bar{x}$	−1.27	−1.32	−1.45	−1.64	−1.74
Contr. 96 Hrs. 28.1° C.	N	6	6	6	6	6
	$\bar{x}$	−1.45	−1.50	−1.49	−1.62	−1.65

are essentially alike, and analyses of covariance (Snedecor, 1956) reported below were used to facilitate these comparisons.

There are at least three confounding factors in the preceding methods. The first two, non-uniform samples and variable warming rate of the brine solution, have been mentioned but the third requires some discussion. The source of dilutant was tap water rather than distilled water because the supply of distilled water was limited. This choice was made deliberately, in full knowledge that the tap water was hard. Salinities of the mixtures were determined by hydrometer, but because many mixtures were measured and averaged in determining the results, the readings were very close to titration values. As an added check, however, samples of the mixtures were taken in capillary tubes and determined by melting point methods, and here departure from expected values became apparent. The greater the dilution the more the melting point was depressed.

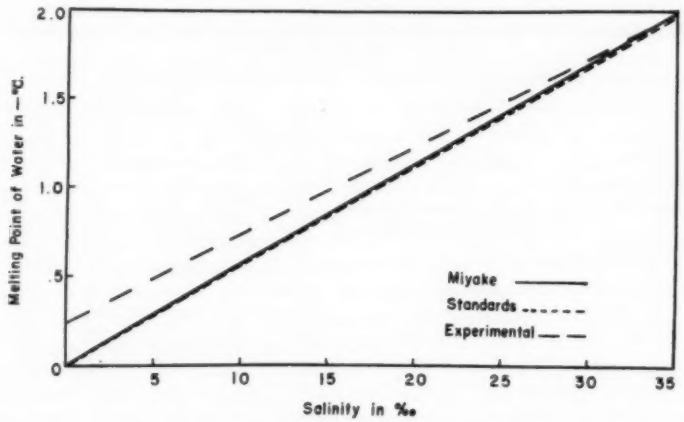

FIGURE 1. Comparison of the relationship between salinity and freezing point depression found by Miyake, that obtained for the standards used in these experiments, and that found in the experimental mixtures of tap water and sea water at average hydrometer-established salinities of 10.06, 15.11, 20.17, 25.09 and 30.50‰.

To determine exactly where the disparity in results lay, the values of Miyake (1939) were used as a basis for reference (Fig. 1). Next to the line for Miyake's values for salinity of west Pacific waters is a line formed by standards mixed for the series of experiments as determined by the differential thermometer. For all practical purposes the lines are identical. A line drawn through mean melting-point values for mixed salinities, however, does not coincide with the first two lines and indeed the deviation appears to be directly proportional to the amount of tap water used in dilution. This disparity is not deemed great and the melting points on the abscissa of Figures 2, 3 and 4 are those determined experimentally and shown in Figure 1. The effect of tap water on osmoregulatory behavior of the shrimp remains unknown.

Results

Juvenile and subadult *aztecus* and *duorarum* are hypotonic to sea water in a salinity range of 30–34‰ in summer in a temperature range of 23–31° C. This was shown by a comparison of the melting points of blood from shrimps and water taken from outside holding tanks at the inception of each weekly experiment. This finding is in accord with the account for a number of decapod crustaceans (*supra cit.*).

Results of six weekly experiments on *aztecus* ranging in total length from 42–100 mm. are shown in Figure 2. Though shrimp in high salinity water prior to experiment are hypotonic, they are hypertonic to hypotonic in the array 10–30‰ after 2–3 hours of immersion in the mixtures at a mean room temperature of 28.3° C., those in the low salinities having more dilute blood than those in high salinities, for the line is not horizontal ($b = .347$). At the end of 48 hours, with an accompanying drop in temperature to 16.2° C., essentially the same relationship exists though blood in low salinities is even more dilute ($b = .464$). Controls at room temperature 28.6° C. show a somewhat similar picture ($b = .512$). At the end of 96 hours

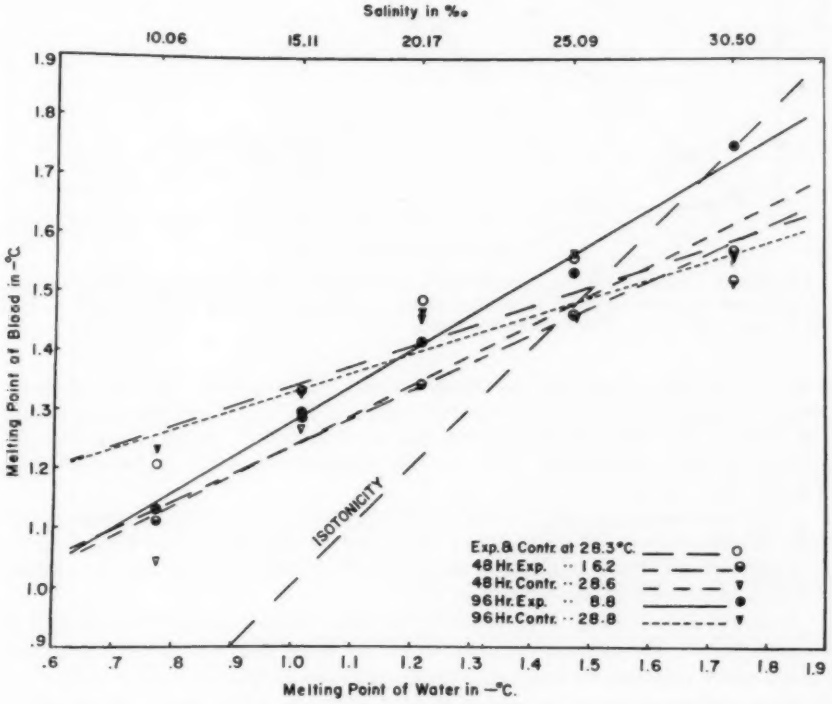

FIGURE 2. *Penaeus aztecus*, 42–100 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of −0.77, −1.01, −1.22, −1.47 and −1.74° C. Key to lines on figure.

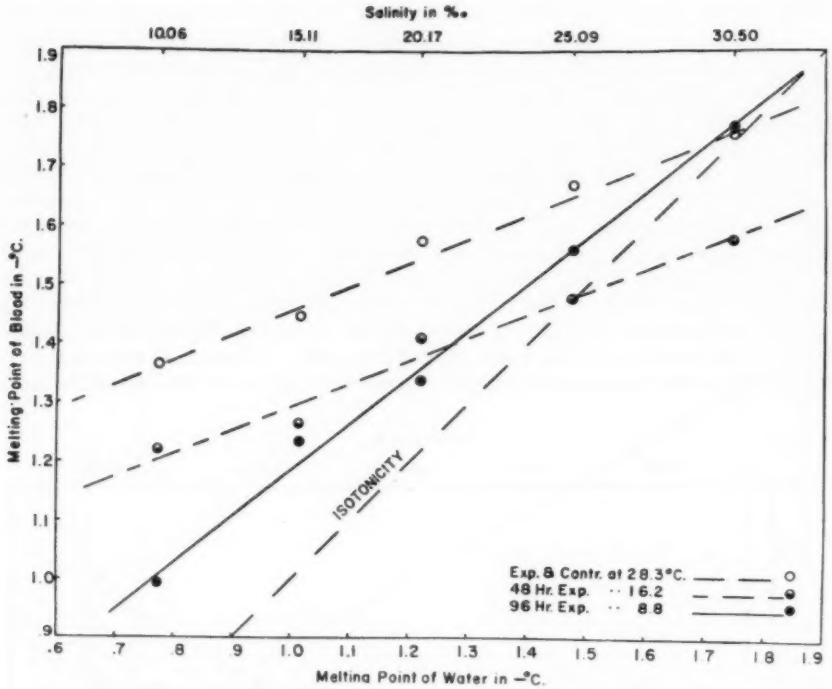

FIGURE 3. *Penaeus aztecus*, 120–150 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of —0.77, —1.01, —1.22, —1.47 and —1.74° C. Key to lines on figure.

at 8.8° C. the slope of the line has changed markedly (b = .613), the low values are nearly the same as those at 48 hours but values in the upper salinities have raised. The slope of the line indicates a trend toward isotonicity. At the same time the controls in room temperature, 28.8° C., have returned essentially to the same point at which they started (b = .323).

Analyses of covariance of data for initial experimental and control animals paired with 48-hour controls, 96-hour controls and experimental animals at 16.2° C. show no significant difference in slope or elevation ($P = 0.05$), but the line for animals in 8.8° C. water differs significantly in slope.

In larger *aztecus* (Fig. 3), essentially the same circumstances prevail though in accented form. The animals hypotonic to the sea water from which they were taken show a quick response to lowered salinities at room temperature (b = .427). At 48 hours with temperature lowered to 16.2° C. the slope of the line is approximately the same as in the beginning but the concentration of all bloods has been lowered (b = .385). At 96 hours the approach of the slope of blood salinities to the line

of isotonicity is even more marked than in the experiments with younger *aztecus* ($b = .783$).

These lines represent averages from only two 96-hour runs; hence less reliance can be placed on them than those of the first experiment and, moreover, there are no controls with which to compare the results. The experiments were done in the hotter weeks of summer when aeration of aquaria was essential, especially when the jars held large animals. During one of the experiments the air-pump for controls stopped and during the other heat was above 30° C. In both cases distress and death among controls was too great to give valid results.

Analyses of covariance of the data for initial experimental and control animals paired with experimental animals at 16.2° C. shows that the slopes are parallel but the elevations differ significantly, and the line for animals in 8.8° C. water differs significantly in slope ($P = 0.05$).

Results of six weekly experiments on *duorarum*, 35–100 mm. total length, are shown as a series of regression lines in Figure 4. Again, animals hypotonic to the external medium at ambient temperature of 28.4° C. quickly make an adjustment

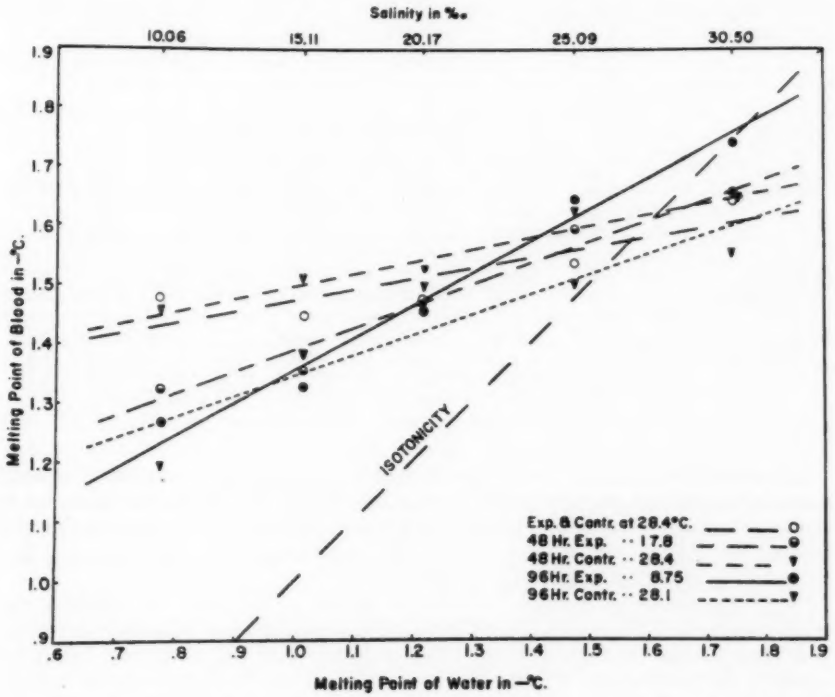

FIGURE 4. *Penaeus duorarum*, 35–100 mm. total length. Regression lines drawn through average melting points of blood at average melting points of water of −0.77, −1.01, −1.22, −1.47 and −1.74° C. Key to lines on figure.

when immersed in an array of 10–30‰ sea water mixtures (b = .182). Within three hours those in dilute media have more dilute blood than those in the less dilute. At the 48-hour point, with temperature gradually depressed to 17.8° C., the slope of this line has changed materially (b = .376). Though animals in 20‰ water have changed little, those in lower salinities have more dilute blood than in the beginning and those in high concentrations have more concentrated blood. Controls at 28.4° C. are essentially unchanged from conditions at the start, but those in higher salinities have somewhat more concentrated blood (b = .210). By the end of 96 hours at a temperature of 8.75° C. a marked change in the slope of the line for blood concentrations is again seen, with a trend toward isotonicity (b = .525). This trend is not so pronounced as in *aztecus* of comparable size (Fig. 2). In controls at 28.1° C. there has been a material dilution of blood at lower salinities (b = .343).

TABLE II

Per cent survival of shrimp at 48 and 96 hours. Forty-eight and 96 hours indicated as mean temperatures for those points. Significant figures in columns for Chi-squares are marked with an asterisk

				Per cent survival						
				Salinity					χ_a^2	χ_b^2
				10‰	15‰	20‰	25‰	30‰		
P. aztecus 42–100 mm.	Exp.	48 Hrs.	16.2° C.	58.8	83.6	90.0	92.5	88.8	17.98*	4.37
		96	8.8°	37.5	77.5	85.0	87.5	80.0	41.55*	10.30*
	Contr.	48	28.6°	70.8	79.2	95.8	91.7	100.0	3.29	1.25
		96	28.8°	45.8	66.6	70.8	83.3	95.8	12.46*	5.42
P. aztecus 120–150 mm.	Exp.	48	16.2°	28.6	64.3	92.9	96.4	96.4	18.08*	3.79
		96	8.8°	7.1	46.4	89.3	78.6	92.9	38.11*	13.97*
P. duorarum 35–100 mm.	Exp.	48	17.8°	67.7	92.5	98.9	98.9	97.8	10.27*	0.59
		96	8.75°	63.4	89.2	97.8	94.6	96.7	13.82*	1.39
	Contr.	48	28.4°	66.7	86.4	91.7	100.0	100.0	3.88	1.21
		96	28.1°	62.5	77.3	91.7	95.8	100.0	5.63	2.25

Analyses of covariance of the data for initial experimental and control animals paired with 48-hour controls and 96-hour controls show no significant difference in slope or elevation ($P = 0.05$); however, the lines representing data for experimental animals at 17.8° C. and 8.75° C. both differ significantly in slope from the line representing the initial animals ($P = 0.05$).

It is instructive to compare survival of shrimp in the array of salinities and temperatures with the results of melting point determinations as expressed in regression lines (Table II, Figs. 2, 3, 4). Some deaths were due to injury, but if we examine the percentages (Table II) it is apparent that poorest survival was in 10‰ salinity. Chi-square (χ_a^2) values for differences in survival among salinities are significant in seven of the ten series ($P = 0.05$), indicating a high influence of salinity on survival. However, if we eliminate the animals so obviously affected by

10‰ salinity from the Chi-square totals (χ_b^2), it is apparent that higher salinities have little effect on survival. The only significant (χ_b^2) values ($P = 0.05$) are for *aztecus* young and adults at 96 hours in 8.8° C. water. This indicates a response to temperature such as suggested by the regression lines in Figures 2 and 3. Further, it is apparent that survival of *duorarum* is on the whole better than young *aztecus,* indicating greater tolerance to lowered salinity and temperature.

Discussion

Melting point determinations show a high degree of individual variation, as has been noted by Gross (1957b) for *Pachygrapsus crassipes.* Such variations may be attributed to stage in the molting cycle, age, sex, and in the present case perhaps to changes in salinity and non-uniformity of sample size. Replications tend to smooth these inequalities.

Each regression coefficient, except one, is significant at the 5% level. The exception is that for *P. duorarum* experimental and control at 28.4° C. and this instance nearly attains significance at the 5% level. The reason for this exception remains unexplained, though it is thought that the average blood melting point value of − 1.48° C. in water with a melting point of − 0.77° C. is low and perhaps not a good approximation.

Both of these species demonstrate possession of osmoregulatory powers. It is apparent that in the size ranges studied both species are hypotonic to sea water, but that in water under 30‰ they are hypertonic. This is in accord with Verwey (1957), who has suggested that it may be a general rule that Crustacea which regulate their internal environments are hypertonic in water of low salinity and hypotonic in water of high salinity.

Under the experimental conditions imposed, both species maintained themselves fairly well for limited periods in a range of 10–30‰ sea water, but with the lowering of temperature the regulatory powers meet more resistance. In such circumstances there appears to be a species difference. Juveniles and subadults of *P. aztecus* demonstrate a loss of osmoregulatory ability with lowering temperature which is statistically demonstrable after 96 hours, though a trend toward lowered activity is apparent even at 48 hours. In *duorarum* a significant difference was manifest in both 48- and 96-hour experimentally cooled samples. From the standpoint of per cent survival, though, indications are that *P. aztecus,* normally only a summer resident in North Carolina, does not regulate in lowered salinities at lowered temperatures as well as does *P. duorarum,* which is normally resident in North Carolina in winter in the juvenile state. The results provide experimental confirmation of information gained from field observations.

The results do not corroborate those of Broekema (1941) that salinity of blood increases with a fall in temperature, with the exception of shrimp in the 30‰ water, but it is possible that longer exposure to lowered temperature in a constant salinity might give different results. Likewise, the results are not in accord with those of Verwey (1957) that though an animal may not maintain a constant internal environment, it does tend to maintain a constant difference between internal and external environment in terms of osmotic pressure expressed in atmospheres at different

temperatures. Again, longer exposure to a given temperature might alter this picture.

In both species it is shown that in all salinities the blood tends to approach isotonicity with the surrounding medium as temperature is lowered.

Summary

1. Melting point determinations on blood of two species of shrimp, *Penaeus aztecus* and *P. duorarum*, were made in an array of salinities of 10.06, 15.11, 20.17, 25.09, and 30.50‰ at 28.1–28.8, 16.2–17.8 and 8.75–8.8° C.

2. These shrimp are hypotonic to sea water at room temperature and hypertonic to dilutions of sea water below 30‰.

3. These shrimp regulate moderately well in experimental dilutions at room temperature, though the blood is diluted somewhat in lowered salinities.

4. At lowered temperatures, 8.75–8.8° C., regulatory ability is impaired and blood tends toward isotonicity.

5. *P. duorarum* is a better regulator at low temperatures than *P. aztecus*.

6. Survival of these shrimp is better in higher salinities at low temperatures.

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